STAIN TECHNIQUE

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BY

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PREFACE

Stain Technique is designed to provide concise instruction in the correct usage of biological stains by introducing the chemistry of color precipitates, the arrangement of stains into a few classes depending on their manner of application, and the outline for staining with combinations of two, three, or four dyes. The results of numerous tests with each dye indicate the action of acids, alkalies, and salts; the composition of effective stains; the influence of reagents on the fastness of color; the affinity for tissues of algae, fungi, mosses, vascular plants, invertebrate and vertebrate animals; and the combination of stains which provide contrasting colors. Because most of the experimental products are of negative value, many of the dyes merit only a brief statement. A few superior and distinctive coal-tar dyes are worthy of admission into standard methods. Hence, the fundamental plan of this textbook is extended to include those phases which assist the student in a practical application of stain technique.

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March 31, 1941

CONTENTS

Chapter I

DYES FIRST USED

During the early investigations of dissected tissues, different chemicals were applied in the hope of finding some means by which the structural details of cells could be seen more clearly under a lens. Frequently, mineral acids were tried; a tincture of iodine was a common reagent. Iodine is still used in the examination of temporary mounts where it colors proteins brown and starches blue. The first organic colors tested as biological stains were the mordant dyes extracted from insects, shellfish, roots, leaves, and wood. The technique inherent in the application of these natural dyes developed slowly because the need and action of mordants was little understood. With the exception of Carmine and Haematoxylin the natural dyes have been replaced in microtechnique by synthetic coal-tar dyes.

Occasionally, coal-tar dyes which are important in a special branch of commercial work are tried in histological procedures. If the results prove satisfactory to the investigator, the dye may be introduced as a special laboratory technique; then the dye is considered a biological stain. The biologist, however, can learn many important facts from an examination of the technique which has been continuously improved by industrial chemists. Large-scale dyeing of wool, silk, and cotton has demonstrated the values and limitations of dyes. Consequently, the manufacturer insists both on a selection of a dye appropriate to each fabric and on a technique that has proven universally effective.

Coloring matters, according to their manner of application are designated as acid, direct, sulfur, mordant, vat, or basic dyes. Acid dyes are used on wool fabrics in a bath containing sulfuric, acetic, or formic acid. Because of their ability to stain cotton without an intervening agent, a few dyes are named direct dyes. On cotton fabrics, sulfur dyes are resistant to the injurious action of alkalies. Naturally occurring dyes require the aid of a mordant including compounds of aluminum, antimony, chromium, iron, tin,

or copper. Frequently, tin salts are employed with Cochineal. For light colors, an aluminum salt is preferred because it does not alter the tint. Chromium and iron compounds, which have a deadening effect on bright tints, are combined with dark colors such as Haematoxylin.

Vat dyes on cotton cloth are very fast to light. They are worked into a paste with a strong alkaline liquor and are reduced to a soluble form with sodium hydrosulfite; this caustic solution is called a vat. Basic dyes provide brilliant colors either on silk, in a weak acid solution, or on cotton with the aid of tannic acid. The latter method of operation suggested the term tannin dyes.

Chapter II

CHEMICAL AND PHYSICAL PROPERTIES OF DYES

Color Precipitates. A discussion of the actions involved in the process of staining may be introduced with examples of color precipitates. A few colloidal pigments give a tissue the same appearance as if a coal-tar dye were used. A blue-black stain such as the tannate of iron is obtained by applying separately either tannic acid and ferrous sulfate, tannic acid and ferric chloride, gallic acid and ferric chloride, or gallic acid and ferric ammonium sulfate. In like manner copper sulfate and potassium ferrocyanide yield brown but ferric salts and potassium ferrocyanide give blue precipitates. It is only through an exchange of radicals between two salts that blue, brown, or black precipitates are produced. A study of the conditions which favor the formation of a permanent pigment in a test tube gives a clue to the correct procedure needed for the application of many biological stains.

Ferric ferrocyanide. A brilliant blue precipitate is formed by mixing equal quantities of 1 per cent ferric ammonium sulfate and 3 per cent potassium ferrocyanide. In the process of staining, the material remains in a solution of potassium ferrocyanide for 15 minutes. After a rinse in distilled water, the tissue is covered with a solution of ferric alum. With the precipitation of ferric ferrocyanide the tissue becomes blue; the color is intensified by repeatedly alternating potassium ferrocyanide with ferric ammonium sulfate because the greater the amount of pigment, the darker is the shade. Prussian Blue is suitable for either glycerin or balsam mounts.

Ferric tannate. The addition of 1 per cent ferric ammonium sulfate to 5 per cent tannic acid yields a blue-black, Hematein-like pigment. An object left in tannic acid for 15 minutes, rinsed with distilled water, and treated with ferric alum becomes blue-black. A repetition of the action with tannic acid and ferric alum increases the amount of ferric tannate.

Hematein. If a drop of ferric alum is added to an aqueous

solution of Haematoxylin, a blue-black precipitate appears. This pigment, Hematein, is the oxidized substance seen in tissues stained with Haematoxylin. In staining, the section is placed first in a solution of ferric alum, whereupon certain types of proteins absorb the iron salt. A rinse in distilled water removes the ferric alum from the surface of the tissue. Now, those parts that have retained the mordant are colored in Haematoxylin; other parts from which the mordant is absent remain colorless.

Reversible actions. In the production of color precipitates, the action remains more or less incomplete because the reverse action also takes place under the same conditions. Since the extent to which the precipitate is affected depends on the concentration of the original salts, it is possible to adjust the conditions in staining and thereby regulate the process in one direction.

With the formation of ferric ferrocyanide, some of the precipitate is dissolved in the presence of an excess of potassium ferrocyanide giving either a suspension of the precipitate or only a colored solution. On the other hand, with a slight excess of ferric ammonium sulfate, the pigment is not dissolved to an appreciable extent.

A tissue left in tannic acid for 15 minutes, rinsed with distilled water, and treated with ferric alum becomes blue-black. By reversing the order of application, using ferric alum first and then tannic acid, some of the iron tannate dissolves in the tannic acid so that within 15 minutes only a part of the original precipitate remains. This Hematein-like pigment is very soluble in tannic acid but less so in ferric alum.

A precipitate forms immediately after a drop of Haematoxylin is added to 1 per cent ferric ammonium sulfate, but the Hematein soon dissolves in ferric alum. In Heidenhain's method of staining, ferric ammonium sulfate is needed first as a mordant. After a rinse in distilled water the tissue is overstained in Haematoxylin; the excess pigment is removed with ferric alum. During the process of destaining the material must be observed under a microscope. As soon as the correct degree of color has been attained, the section is taken out of the ferric alum, but the reversible action

continues until the section is washed with distilled water. After a second rinse, in distilled water containing a trace of ammonium hydroxide, the oxidation of Hematein is completed and the object is stained deep blue-black. If the material is washed with tap water containing sulfuric acid, the pigment is greenish black.

Oxidation. In microtechnique, the study of the conditions which effect color is especially important; the behavior of biological stains differs with the physical and chemical properties of the tissues and with the presence of chemical assistants. Many coal-tar colors are more fast if they are developed by means of oxidation. The importance of this reaction can be demonstrated with a number of azo dyes which do not stain plant tissues unless an oxidizing agent is added to the dye bath. If Erythrosin, a Xanthene dye, is absorbed by a tissue in the presence of potassium dichromate, the pink is changed to scarlet. With many of the coal-tar dyes, however, the oxidized color is soluble in water or alcohol; consequently such stains are unsuited for permanent mounts.

In aqueous solutions of Picric Acid, plant and animal tissues remain pale yellow. With the addition of acetic acid to the stain, the yellow is increased but fades after five hours exposure to sunlight. With the aid of potassium dichromate and acetic acid, the color changes very little after more than 50 hours exposure to sunlight. Thus, the presence of appropriate chemical assistants strikingly modifies the fastness of Picric Acid.

Reduction. Dyes may be affected by reduction in a number of ways. In a glycerin mount, ferric ferrocyanide fades to white ferrous ferrocyanide unless a trace of potassium dichromate is added to the dilute glycerin during dehydration. Most of the sulfur, mordant, and vat dyes insoluble in water or alcohol are brought into solution by reduction. After the tissue has retained the stain in its soluble state, the color is developed by re-oxidation. The oxygen necessary to complete the action may be absorbed from the air. Hydrochloric acid in acid-alcohol changes many basic dyes to the leuco compound, the form of dye which is not necessarily colorless but which is usually more fugitive. For this reason, the

destaining of basic dyes with acid-alcohol has been omitted from the technique described in this text.

Physical properties. In a few staining procedures the absorption of color is entirely physical. If sections of germinating corn grains remain in a solution of Sudan IV, the tissue containing fats stains a bright red. The dye is absorbed by the fats but not by the proteins or carbohydrates. Here, the oil soluble dye, unchanged chemically, is held in suspension. The dye is extracted from the fatty tissue as soon as the stained sections are changed to strong alcohol, acetone, or xylene. In a similar manner, a dye dissolved in an oil such as clove oil or aniline oil is retained by a tissue in the same form as it occurs in the oil suspension. Colors produced in this manner are not oxidized pigments; they disappear gradually or dissolve in balsam.

Chapter III

GENERAL USES OF BIOLOGICAL STAINS

Biological stains are prepared from powdered dyes. Solution is effected in water or dilute alcohol with the addition of chemical assistants. · In commercial dyeing certain chemicals are used both as levelling agents to develop an even shade and as assistants to exhaust the dye bath or to fix the dye to the fabric. Any substance added to a biological stain for the purpose of producing a better differentiation of color may be considered a chemical assistant.

A trace of sulfuric acid is added to acid dyes to increase the staining action, provided precipitation does not occur; with basic dyes acetic acid retards the staining process. Should a dye be precipitated by sulfuric acid, the effective sulfate ion is obtained from sodium sulfate and the acid reaction from a weaker acid such as acetic or formic acid. Whenever the dye is sensitive to all acids, the oxidizing agent potassium dichromate is tried. Ammonium alum is added to basic and direct dyes because the color becomes more fast to washing. The sulfur and vat dyes are insoluble in dilute acids but soluble in weak alkalies.

Chemicals necessary in the formation of color precipitates are seldom added to the stain but are applied in separate solutions. The salts of heavy metals especially aluminum, iron, and chromium combine with mordant dyes to give insoluble color lakes. The need for a chemical assistant is more clearly demonstrated with a mordant dye than with a basic, acid, or direct dye.

The active biological stains are separated into two large classes acid dyes and basic dyes. The basic dyes form a homogeneous group; one type formula is sufficient for the stains used on either whole mounts or paraffin sections. Although the acid dyes produce similar results on a particular tissue they differ in their manner of application. These differences are indicated in the subdivisions acid, direct, sulfur, mordant, and vat dyes.

Acid dyes, Colour Index Numbers.

151. Orange II.
252. Crocein Scarlet MOO.
280. Biebrich Scarlet.
286. Crocein Scarlet 7B.
692. Acid Fuchsine.
707. Aniline Blue.

Many of the acid dyes are sodium salts of a sulfonic acid. The principal salt-forming radical is the hydroxyl group. The radical $-SO_3Na$, which is not considered an auxochrome, renders the dye more soluble in water and more acidic. If possible, 0.01 per cent sulfuric acid (1 ml. of 1 per cent) should be added to each 100 ml. of the dye solution. A few dyes are precipitated by sulfuric acid, whereupon either acetic acid, formic acid, sodium sulfate, or potassium dichromate becomes the chemical assistant. With many of these dyes the amount of ionic dissociation of the molecule in water is so slight that the stain is relatively inactive. The intensity of the stain is increased by the presence of an acid, decreased with an alkali.

The routine procedure with an unfamiliar acid dye requires three tests. The first object taken from the stain at the end of 15 minutes is dehydrated in alcohol and cleared in xylene. An examination of the finished product shows whether or not a 15-minute interval is sufficient. If the color is too light, the second section is tested at the end of an hour. The third section, in turn, remains in the stain 12 to 24 hours. The activity of acid dyes does not extend beyond 24 hours. Naturally, if the first test at the end of 15 minutes gives too intense a color a shorter interval is indicated.

Acid dyes are especially suited for proteins, either in plant or animal tissues. Within plants the proteins are more abundant in parenchyma, cambium, phloem, and immature spores. It is impossible to give a definite time limit for any particular dye because the time differs both with the kind of tissue and with the fixative. Orange II is sometimes too weak to color mature parenchyma in 24 hours, yet the meristem of root tips fixed in sublimate-dichromate-acetic is deeply stained within a few seconds. On animal tissues, Orange II requires an average of three to five minutes although sections may be found that are not overstained in 24 hours.

Direct dyes, Colour Index Numbers.

327. Direct Fast Scarlet 4BS.
370. Congo Red.
518. Niagara Sky Blue 6B.
520. Niagara Sky Blue.
620. Sun Yellow.
957. Sulfur Brilliant Blue.
1006. Sulfur Green.
1012. Sulfur Bordeaux.

The principal radicals of direct dyes are $-NH_2$ and $-OH$, the auxochromes that characterize the basic and acid dyes respectively. A direct dye is applied in neutral solutions containing sodium sulfate, in $\frac{1}{2}$ per cent acetic acid, or in alkaline solutions. Direct dyes containing alkalies cannot be used on paraffin sections but they are often valuable for the coloring of minute objects mounted entire in glycerin. Solutions of Direct Fast Scarlet 4BS, Niagara Sky Blue, and Sun Yellow should contain both acetic acid and sodium sulfate as chemical assistants. Because Congo Red changes from red to blue in the presence of an acid, only sodium sulfate is used in the stain. The direct dyes provide contrast colors with acid dyes in the staining of animal tissues. Direct dyes are similar to acid dyes and stain the proteins of connective tissues, muscles, blood corpuscles, fungous mycelia, and parenchyma.

Sulfur dyes, which are direct dyes containing sulfur, are soluble in alkaline solutions of sodium sulfide. They are especially suited to the staining of blue-green algae. These pigments are insoluble in glycerin, whereas the colors of most acid and basic dyes are extracted by this medium. Since the sulfur dyes are decolorized by alcohol or acids they are not used on paraffin sections.

Mordant dyes, Colour Index Numbers.

781. Gallein (acid).
883. Gallocyanine (direct).
1049. Acid Alizarin Green G (acid).
1051. Alizarin Cyanine G (direct).
1239. Carmine (acid).
1246. Haematoxylin (acid).

The behavior of mordant dyes parallels the differences noted between acid and direct dyes. The acid mordant dyes stain tissues

taken from a fixative containing the salt of a heavy metal, mercuric chloride, chromic acid, or potassium dichromate. The direct mordant dyes have a more extensive application because they are not adversely affected by fixatives. Sections of paraffin materials are left in 1 per cent ferric alum or 5 per cent ammonium alum for 30 minutes to an hour. After rinsing in distilled water, the section is covered with the stain. Some of these dyes are inactive until they are heated to 50° C. The depth of color in a finished mount may be estimated from the section in water; dehydration in alcohol extracts very little of the color. The time required in a mordant dye differs: (1) Gallein one to ten minutes, (2) Gallocyanine one minute to an hour, (3) Acid Alizarine Green G five minutes to an hour, (4) Alizarine Cyanine G one minute to ten hours, (5) Carmine 30 minutes to 24 hours, (6) alum Haematoxylin 15 to 30 minutes, and (7) iron Haematoxylin one to three minutes or sometimes as long as 24 hours.

Vat dyes, as a class, are very fast to light. Their application is limited, at present, to the staining of blue-green algae, page 64.

Basic dyes, Colour Index Numbers.

680. Methyl Violet.
740. Acridine Red.
752. Rhodamine 6G.
841. Safranine.
924. Methylene Green.
927. New Methylene Blue.

All of these dyes are soluble in water. The stain should contain 0.5 per cent of the dye, except Methyl Violet where only 0.1 per cent is needed. Acetic acid and ammonium alum are the chemical assistants.

The characteristic properties of basic dyes are derived from the radicals $-NH_2$, $-N(CH_3)_2$, or $-N(C_2H_5)_2$. They are usually marketed in the form of hydrochloride salts. In general, they stain plant tissues 25 times more rapidly than the acid dyes. The rate at which the staining process takes place depends on the extent of ionization of the dye molecule. In the presence of a small quantity of alkalies the color reaction is intensified, as in the staining of bacteria with alkaline Methylene Blue. The presence of

an acid retards the speed of staining, ammonium alum produces a color more fast to washing, and the two together in the stain provide a better differentiation of minute cell structures. Basic dyes have an affinity for tissues containing tannins, resins or fatty acids; for spores, lignified xylem, cellulose, fibers, and cork. The chromatin and chromosomes are stained before the cytoplasm is affected.

An unfamiliar basic dye is used for one minute. If the color is too light, try one hour and then 24 hours. Whenever the color is too dark at the end of one minute, leave the material in the stain for five seconds. With two or three tests it is possible to approximate the time interval that gives the desired result. A clear color is obtained with Rhodamine 6G in 12 to 24 hours, Safranine in 15 minutes to 24 hours, Methylene Green 15 to 30 minutes, New Methylene Blue in five to ten minutes, and Methyl Violet in three seconds to ten minutes.

Nomenclature of dyes. Under the above system of classifying the commercial dyes, a very convenient mode of indicating the particular shade of dyestuff by a letter or number denotes: A, extra concentrated, as in Safranine A; B, blue, as in Niagara Sky Blue 6B; D, direct, in Alkaline Green D; G, (gelb) yellow, as in Pyronine 2G; O, concentrated, in Safranine O; R, red, an Acridine Yellow R; S, (sauer) acid, as in Fuchsine S; X, concentrated, in Safranine X; Y, yellow, as in Bismarck Brown Y; and the numerals, I, II, III, in Orange I, Orange II, Orange III, and Orange IV which indicate a series of shades of one color.

Chapter IV

STAINING WITH CONTRAST DYES

The correlation between tissue elements and particular classes of dyes serves as a working basis for the selection of combinations of contrast dyes. The composition of organic materials may be regarded as comprehensive groups of carbohydrates, carbohydrates and proteins, or only proteins. Carbohydrates are stained more readily with basic dyes but proteins are differentiated more easily with various types of acid dyes. This initial relation between tissues and stains implies: (1) acid dyes for embryonic or mature animal tissues and meristematic parenchyma, (2) basic and acid dyes for stem, root, or leaf, and (3) basic dyes for sections of wood. As a general rule the greater the inherent difference in cellular structure the greater should be the chemical differences of the dyes present in the combination.

The selection of any combination of two or more dyes depends not so much on whether the section is animal or plant tissue as on whether the section consists of similar or unlike substances. In most sections of animal tissues, fungi, and meristems, the variations are usually between types of proteins. These similar substances provide different colors more easily in mixtures of closely related acid dyes. Although the color of nuclei may be intensified with basic stains, clear contrasts of protein elements are better in acid dyes with basic properties, that is, direct dyes which possess an amino radical. Whenever cellulose assumes a dominant part of vascular plants a basic dye is necessary. Then, the red basic Rhodamine 6G, Safranine, Acridine Red, or Basic Fuchsine may be used with a counterstain such as Aniline Blue, Niagara Sky Blue, Fast Green, or Haematoxylin.

Many stains either lack a suitable counterstain or they differentiate with few other dyes. Sun Yellow, a direct dye, gives a clear contrast with Niagara Sky Blue or New Methylene Blue, but blends on plant tissues into a dull gray-violet with Methyl Violet. Biebrich Scarlet, an acid dye, differentiates sharply with either of the blue dyes or the violet basic dye.

Selecting a combination of stains. Test the object with a stain from each of the four classes: acid, direct, mordant, and basic. If the best colors are given with basic and mordant stains, the combination of Safranine and Haematoxylin may be tried. Should the direct dye work better than Haematoxylin, use Safranine and Niagara Sky Blue. Many sections of growing tissues fail to differentiate with a combination containing a basic dye. If both the acid and direct stains provide clear colors on the test material, the desired contrast is obtained by using either an acid and a direct dye or two acid dyes of contrasting colors. The direct dyes may be used as contrast stains with either an acid or a basic dye.

Combinations of two stains.

Rule: Use the lighter stain first.

Example No. 1—Crocein Scarlet MOO, C. I. No. 252, and Niagara Sky Blue, C. I. No. 520.

This red acid dye and blue direct dye provide an adequate contrast on small invertebrates, paraffin sections of animal tissues, meristems, or on fungi. The combination of stains may be used in two ways, either separately or mixed in solution.

I. *Two dyes used separately.*

1. Ten minutes in Crocein Scarlet MOO.
2. Rinse the material in distilled water.
3. One minute in Niagara Sky Blue.
4. Rinse the material in distilled water.
5. Objects used as whole mounts are given three minutes in each grade of alcohol: 35, 50, 70, 85, 95, and absolute alcohol. Paraffin sections are transferred from water to 95 per cent alcohol and then into absolute alcohol, one minute in each.
6. Materials used as whole mounts need three minutes in each mixture: $\frac{1}{4}$ xylene, $\frac{1}{2}$ xylene, $\frac{3}{4}$ xylene and xylene. Paraffin sections are given one, two, three, and five minutes respectively in the alcohol-xylene mixtures.
7. Whole mounts are infiltrated gradually in balsam.

Cleared sections are covered with a drop of balsam and a dry cover glass is added.

II. *Two dye-solutions mixed.*

1. Five minutes in a mixture of ten parts Crocein Scarlet MOO and one part Niagara Sky Blue.
2. Rinse the material in distilled water.
3. Follow the instructions given in the previous outline schedule beginning with dehydration.

Example No. 2—Sun Yellow, C. I. No. 620, and New Methylene Blue, C. I. No. 927.

This combination of a direct yellow and a basic blue dye is especially useful on plant tissues; it may be tried on invertebrate animals.

1. The material is given 24 hours in Sun Yellow.
2. Rinse the objects in distilled water.
3. Counterstain in New Methylene Blue, five minutes.
4. Rinse the material in distilled water.
5. Follow the usual laboratory procedure beginning with dehydration.

The two dyes cannot be mixed because a precipitate forms as soon as the stains are combined. The length of time the tissue remains in New Methylene Blue modifies the results giving either yellow and green, green and blue, or the three colors, yellow, green, and blue.

Example No. 3—Rhodamine 6G, C. I. No. 752, and Aniline Blue, C. I. No. 707.

In staining mature vascular plants, the tissues are left for 24 hours in Rhodamine 6G, a bright red basic dye that does not overstain. The principal problem of technique at this stage is the addition of enough Aniline Blue so that the red and blue are of equal density. Use Aniline Blue on one stem section for a minute, on another for two minutes, and on a third for five minutes. These three sections in xylene serve as a comparison in determining the correct time interval to be used.

If a counterstain covers all of the first color within a few seconds, the counterstain is too intense. Under this circumstance, a

weaker counterstain should be tried or the first stain should be changed to a more intense dye of the same color. Blue dyes that do not act so rapidly as Aniline Blue are Niagara Sky Blue and alum Haematoxylin. If Rhodamine 6G does not give enough color, the more intense Safranine or Acridine Red should be tried.

Combinations of three stains. The technique of applying three of four dyes is nearly the same as that employed with combinations of two stains. Only two solutions are needed when two or more dyes from the same group, either acid or basic, are mixed and used as one stain. The proportionate amount of each dye in a mixed stain is calculated either by the spot test or the time test.

1. *Spot test using basic dyes.* On filter paper note the contrast between the red from a drop of Safranine and the violet from a drop of Methyl Violet. For the first test try equal quantities of Safranine and Methyl Violet; compare a drop of this mixed stain with the spots of the separate dyes on the filter paper. If the spot given by a drop of mixed dyes shows a color intermediate between the colors of the two original stains, the mixture contains the proper amount of each. Should one color predominate in the test on paper, the proportions are changed until the result is a blend of the two original colors. As a rule, three parts of Safranine will neutralize one part of Methyl Violet.

Staining procedure for mature plant tissues. Stain the material for 24 hours in Orange II, rinse in distilled water, and follow with the mixture of three parts Safranine and one part Methyl Violet. Again rinse the section in distilled water, transfer it to 95 per cent alcohol, complete the dehydration in absolute alcohol, and clear in xylene. During the counterstaining, a longer interval in the mixed stain yields more violet while less time increases the red.

2. *Time test using acid dyes.* Note the different time intervals needed for staining a section of an earthworm in Sun Yellow, Orange II, and other acid dyes.

Sun Yellow, C. I. No. 620, 10 minutes.
Orange II, C. I. No. 151, 5 minutes.
Crocein Scarlet MOO, C. I. No. 252, 5 minutes.
Biebrich Scarlet, C. I. No. 280, 2 minutes.

Crocein Scarlet 7B, C. I. No. 286, 2 minutes.
Acid Fuchsine, C. I. No. 692, 30 seconds to 1 minute.

Whenever two of these dyes are mixed the amounts needed are in direct proportion to the time interval required for the staining of each alone. Thus, mix ten parts of Sun Yellow with five parts of Orange II, ten parts of Sun Yellow with one part of Acid Fuchsine, or five parts of Orange II with one part of Acid Fuchsine. In the following outline any of the eight mixed stains may be tried with one of the three counterstains.

Mixed stains	*Counterstains*
Sun Yellow (10) and Orange II (5)	(1) Aniline Blue
Sun Yellow (10) and Crocein Scarlet MOO (5)	(2) Niagara Sky Blue
Sun Yellow (10) and Biebrich Scarlet (2)	(3) New Methylene Blue
Sun Yellow (10) and Crocein Scarlet 7B (2)	
Sun Yellow (10) and Acid Fuchsine (1)	
Orange II (5) and Biebrich Scarlet (2)	
Orange II (5) and Crocein Scarlet 7B (2)	
Orange II (5 to 10) and Acid Fuchsine (1)	

Staining procedure. Using sections of an earthworm, try one of the mixed stains such as Sun Yellow and Orange II for five to ten minutes. If the first mixed stain does not produce two colors on the section, try another mixture. When two colors are obtained, the mixture may be used with a counterstain. The problem at this point is to find which of the three blue counterstains will give the best result; it is taken for granted that from sufficient experience in handling double stains the principle involved in the procedure of counterstaining is understood.

a) *On animal tissues.* Try the mixed stain for five minutes, rinse the section in distilled water, and counterstain in Niagara Sky Blue for one minute or in Aniline Blue for 30 seconds; it may be necessary to shorten or to increase the time in Niagara Sky Blue. Any one of the eight mixed stains may be tried on sections of earthworm and counterstained in either Niagara Sky Blue or in Aniline Blue.

b) *On meristematic plant tissues.* Use the mixed stain for one hour, rinse in distilled water, and counterstain in one of the blue dyes for one minute.

c) *On mature plant tissues.* Use the mixed stain for 24 hours. If there is an abundance of cellulose in the section, New Methylene Blue for five minutes is the best counterstain. On fungous tissues either Niagara Sky Blue or Aniline Blue gives a better contrast than New Methylene Blue.

Combinations of four stains.

Mixed stains	*Counterstains*
Sun Yellow (10), Orange II (5), and Biebrich Scarlet (2)	(1) Aniline Blue
Sun Yellow (10), Orange II (5), and Crocein Scarlet 7B (2)	(2) Niagara Sky Blue
Sun Yellow (10), Orange II (5), and Acid Fuchsine (1)	(3) New Methylene Blue

Staining procedure. On invertebrate animals use the first-mentioned mixed stain for five minutes and counterstain either in Niagara Sky Blue for 30 seconds or in Aniline Blue for 15 seconds. On sections of vertebrate tissues the second- or third-listed stain may be tried for five minutes; counterstain either in Niagara Sky Blue or in Aniline Blue.

On sections of mature plant tissues use the first or third mixture for 24 hours and counterstain in New Methylene Blue for five minutes.

A combination of four dyes should be used only on appropriate materials consisting of a mass of different tissues. Whenever the object presents a uniform structure one or two stains may be sufficient. In searching for a better technique, keep in mind the correlation between dyes and tissue elements. Note the differences between acid, direct, mordant, or basic stains and determine the adaptability of the material by testing separately each of the dyes present in the combination.

Chapter V

BIOLOGICAL STAINS

A. Synthetic Organic Dyes, Classes I to VI

The synthetic coal-tar colors are classified on the basis of molecular structure. The terminology of the functional parts of the dye molecule is that proposed by Otto N. Witt in 1876. The simplest compounds which illustrate the evolution of a dye are derived from benzene. Particular radicals capable of imparting color to aromatic compounds are called chromophores. The chromophore $-NO_2$ added to a molecule of benzene yields nitrobenzene, a chromogen. The color-bearing nitrobenzene does not possess staining properties until it is transformed into a compound capable of electrolytic dissociation. Salt-forming groups called auxochromes, either hydroxyl or amino radicals, change an inert chromogen to an acid or a basic dye respectively. Nitrophenol is a simple acid dye containing the chromophore $-NO_2$ and the auxochrome $-OH$; nitroaniline is a simple basic dye including the chromophore $-NO_2$ and the auxochrome $-NH_2$.

The discussion of the separate dyes specifies the *Colour Index* Number, the name most frequently used in American literature, synonyms, fastness to light, the formula for the stain, and the manner of application. The arrangement of the dyes follows the classification of dyestuffs as given in the *Colour Index,* published by the Society of Dyers and Colourists, Bradford, England, 1924. Each coal-tar dye is given a separate number and is placed in one of 26 classes according to the kind of chromophore in the dye molecule; the dye of the simplest structure is first in each class.

The comparative fastness of each dye is given on a scale of 1 to 5 for paraffin sections ten microns thick. A fastness of 1 indicates a color that does not fade after 50 hours exposure to direct sunlight at midday during the summer months. For the value 2, the color fades slowly but lasts more than 50 hours on exposure to sunlight. In group 3, the color disappears within 50 hours although it is still evident at the end of 25 hours. Pigments that fade within

25 hours are placed in group 4. Group 5 includes extremely fugitive colors. Dyes with a light fastness of 4 or 5 have little value for permanent slides.

Class I. Nitroso Colouring Matters

5. **Naphthol Green B** is an acid dye, fastness to light 2; ½ gram in 100 ml. of distilled water and 1 ml. of 1 per cent sulfuric acid. In commercial dyeing the nitroso dyes are applied using an iron salt in the dye bath but in staining tissues a darker green is obtained by using the iron salt separately as if it were a mordant. A deficiency of tinctorial properties limits the use of Naphthol Green B on paraffin sections except in plant tissues consisting of dense spore masses.

Mordant the sections of an aethalium of *Fuligo,* a Myxomycete, in 1 per cent ferric ammonium sulfate for 30 minutes, rinse in distilled water, and stain for from one to 24 hours. On whole mounts of fungous mycelia five to ten minutes is sufficient with ferric alum as a mordant, 24 hours without the mordant.

Obelia, Sertularia, Hydra, and animals of similar size are placed in 1 per cent ferric alum for 30 minutes. After a rinse in distilled water the objects are stained dark green in ten minutes; thick specimens are easily overstained. Without the iron alum mordant six to ten hours is needed. The invertebrates stained in Naphthol Green B ten minutes may be counterstained, after a rinse in distilled water, for 15 minutes in Direct Fast Scarlet 4BS, C. I. No. 327.

Class II. Nitro Colouring Matters

7. **Picric Acid,** trinitrophenol, is an acid dye, light 2 to 5; ½ gram in 95 ml. of distilled water, 5 ml. of ½ per cent potassium dichromate, and ½ ml. of acetic acid. Although Picric Acid is the oldest of the artificial dyestuffs it is rarely used commercially as a dye except in small quantities in mixtures for shading. From an aqueous stain animal and plant tissues are pale yellow. With the aid of potassium dichromate and acetic acid the newly developed pigment appears a fast yellow which, in most tissues, is too light.

9. **Martius Yellow,** Aniline Yellow, Naphthylamine Yellow is

an acid dye, light 4 to 5. The absence of a bitter taste accounts for the former use of this poisonous dye in coloring starchy food such as vermicelli and macaroni. Pianese includes Martius Yellow with Malachite Green and Acid Fuchsine for the differential staining of cancerous tumors. Other technicians use the triple stain for fungous parasites on plants. There is no need for Martius Yellow in the mixture when the yellow can be traced to either a chemical in the fixative or a natural pigment. Instead of Martius Yellow use Sun Yellow, C. I. No. 620.

10. **Naphthol Yellow S,** Acid Yellow is an acid dye, light 3 to 4. Only a light color is obtained unless acetic acid and potassium dichromate are present in the stain. It is more fast than Martius Yellow and more soluble in water; acids cause no precipitates in its solution. Its principal value is for the coloring of foods such as noodles and macaroni.

Class III. Monoazo Colouring Matters

Although the azo chromophore is basic in character, the azo dyes may be either basic or acid according to the auxochromes present. The number of chromophores in the dye molecule determines the classification as monoazo, disazo, trisazo, or polyazo dyes. Those azo dyes which are insoluble in water or alcohol may be used as fat soluble stains. Azo dyes differ through all degrees of fastness to light; many are fugitive whereas others can satisfy the most exacting requirements in this respect. In some instances the addition of potassium dichromate to the stain increases the intensity of the color and its fastness.

16. **Acid Yellow,** Fast Yellow S is a direct dye, light 2 to 3; $\frac{1}{2}$ gram in 95 ml. of distilled water, 1 ml. of 1 per cent sulfuric acid, and 5 ml. of $\frac{1}{2}$ per cent potassium dichromate; filter. Sections of animal or plant materials are stained a clear fast orange-brown that is not very brilliant.

20. **Chrysoidine Y,** and 21. **Chrysoidine R** are basic dyes, light 4; the former dye is more soluble. The stain decomposes slowly on standing. These dyes are similar to Bismarck Brown in color but they are not its equal in fastness. Sharp contrasts are

obtained by counterstaining with direct blue dyes such as Niagara Sky Blue.

27. **Orange G,** Acid Orange G is an acid dye, light 3 to 4; ½ gram in 100 ml. of distilled water and 1 ml. of 1 per cent sulfuric acid. The fastness of Orange G to washing is greatly increased by the presence of acids. If the material is treated with ½ per cent potassium dichromate, the stain reacts more rapidly; potassium dichromate cannot be added to the dye. Best results occur on tissues fixed in a solution containing picric acid; with other types of fixatives Orange II is better.

Orange G is used extensively on animal tissues as a counterstain in conjunction with iron Haematoxylin. Because the iron alum present in the tissues as a mordant for Haematoxylin also serves as a chemical assistant for Orange G, the stain need not contain sulfuric acid. Since the orange tends to obscure a portion of the finer details of cell structure, many technicians prefer iron Haematoxylin without a counterstain.

Mallory's connective tissue stain includes Orange G, Aniline Blue, and Acid Fuchsine. On many sections, Aniline Blue fails to differentiate well with Orange G; the colors may be striking but they usually lack clear definition. The deficiencies inherent in Mallory's triple stain are also found in sections stained with Ehrlich's Orange G, Methyl Green, and Acid Fuchsine.

A more satisfactory combination of stains is a mixture of nine parts Orange G and one part Acid Fuchsine for two minutes which is followed by Aniline Blue for 15 seconds on most sections of animal tissues. As an alternative procedure try Orange II and Acid Fuchsine followed by Niagara Sky Blue.

Flemming's triple stain is frequently used on all kinds of plant tissues. Safranine is applied first; the excess of red is removed in acid-alcohol. A brief stain in Gentian Violet is followed by dehydration and then Orange G in clove oil is added. These three dyes may be applied in a simpler manner. Stain the plant tissue in Orange G, or preferably Orange II, and then apply the counterstains in a mixture of three parts Safranine and one part Methyl Violet, page 21.

In the contrast staining of plant tissues mordant the material for 15 minutes in ½ per cent potassium dichromate, rinse in distilled water, use Orange G overnight, rinse again with distilled water, and follow with: (1) one minute in Methyl Violet, C. I. No. 680, (2) five minutes in Niagara Sky Blue, C. I. No. 520, or (3) five minutes in New Methylene Blue, C. I. No. 927.

28. **Ponceau G,** 2G, Scarlet 2G is an acid dye, light 3 to 4; ½ gram in 100 ml. of distilled water and 1 ml. of 1 per cent sulfuric acid. Many tissues are stained fuchsia. Materials previously treated with potassium dichromate are scarlet and more fast to washing. One of the "poppy" dyes, it is similar to Ponceau 3R, C. I. No. 80, and Crocein Scarlet MOO, C. I. No. 252. On animal tissues use Ponceau G in the same manner as described in the technique for Crocein Scarlet MOO.

On plant tissues use the stain for 12 to 24 hours and follow with: (1) Victoria Blue B, C. I. No. 729, five minutes for reddish brown and blue, (2) Methyl Violet, C. I. No. 680, one minute, or (3) Niagara Sky Blue, C. I. No. 520, five minutes for reddish brown and blue.

29. **Chromotrope 2R** is an acid dye, light 4. There are many acid dyes of a nondescript fuchsia; this one is very fugitive to light. As successive azo dyes are considered notice the progressive increase of color intensity.

30. **Azofuchsine,** Acid Azofuchsine is an acid dye, light 3. The bright fuchsia obtained on plant tissues is changed to bordeaux with either a mordant or an after-treatment of potassium dichromate.

31. **Pontacyl Carmine 2G** (Du Pont) is an acid dye, light 3. This fuchsia pigment is more intense than the color obtained with Azofuchsine. After a potassium dichromate mordant, the color changes to bordeaux. Fungous mycelia treated with a mordant remain light fuchsia.

36. **Alizarin Yellow G,** and 40. **Alizarin Yellow R** are mordant dyes, light 5. The color on paraffin sections is fugitive. The alcoholic solution of the dye stains blue-green algae a brilliant yel-

low-brown that gradually disappears when the algae are transferred to glycerin.

44. **Para Red,** Azophor Red PN is a developed dye which is not adaptable to paraffin sections. It may be produced on whole mounts by first using an alkaline beta-naphthol solution and then following with an ice cold diazotized paranitroaniline. The orange-red pigment lacks sufficient density for whole mounts. Because Azophor Red is insoluble in water or alcohol, it cannot be used as a stain.

57. **Pontacyl Carmine 6B** (Du Pont) is an acid dye, light 3. Thin sections of tissue are fuchsia with a decided bluish cast. A potassium dichromate mordant darkens the color considerably. Numerous tints and shades which are valued in commercial dyeing are not so important in microtechnique where the bright primary colors are preferred.

73. **Sudan II** may be used as a fat stain; 1/10 gram in 50 ml. of 95 per cent alcohol and 50 ml. of 1 per cent sulfuric acid. Freehand sections of fresh tissue containing fat become orange-red at 50° C. although starches and proteins remain unchanged.

74. **Scarlet R** is an acid dye, insoluble in water. Because it is soluble only in alkaline 50 per cent alcohol, the dye is unsuited for staining paraffin sections.

79. **Ponceau R,** 2R, Scarlet R, 2R is an acid dye, light 4. Plant tissues are stained a bright red that contrast well with green basic dyes. It is similar to Ponceau 3R which is more fast. Animal tissues are stained in the same manner as described in the technique for Ponceau 3R, C. I. No. 80.

80. **Ponceau 3R** is an acid dye, light 3; ½ gram in 100 ml. of distilled water and 1 ml. of 1 per cent sulfuric acid. On sections of animal tissues use a mixture of three parts Sun Yellow, C. I. No. 620, and one part Ponceau 3R for five minutes; follow with (1) Niagara Sky Blue, C. I. No. 520, for two minutes or (2) with Aniline Blue, C. I. No. 707, one minute. The results are not satisfactory on a tissue that has been fixed in either picric acid or a chromium salt.

On plant tissues the scarlet is similar to that of Biebrich Scarlet. Leave the material in Ponceau 3R for 12 hours and counterstain with one of the following: (1) five minutes in Victoria Blue B, C. I. No. 729, (2) five minutes in Brilliant Cresyl Blue, C. I. No. 877, for reddish brown and violet, or (3) one minute in Methyl Violet, C. I. No. 680, for fuchsia and violet.

88. **Bordeaux,** Azo Bordeaux, Acid Bordeaux is an acid dye, light 3; $\frac{1}{2}$ gram in 100 ml. of distilled water and 1 ml. of 1 per cent sulfuric acid. The fuchsia on plant materials blends with blue dyes to give colors of little contrast; a fair differentiation is obtained with Victoria Blue B, C. I. No. 729. Bordeaux is also used for coloring foods.

101. **Pontachrome Brown MW** (Du Pont) is a mordant dye, light 4. A clear brown of moderate fastness is obtained on blue-green algae with the alkaline solution of the dye. Neutral solutions in alcohol give a reddish brown on paraffin sections.

120. **Coccinine** is an acid dye, light 3 to 4. Materials in paraffin sections are stained a pale pink which is not the equal of Biebrich Scarlet or of the Crocein Scarlets.

133. **Janus Green B** is a basic dye, light 4. This dye, now obsolete, was formerly used on fabrics. With staining properties decidedly different from most basic dyes, it can be applied on cotton in an acid bath. In whole mounts of algae the stain is polychromatic; in a stem section all of the cells are either blue or blue-green. If it were more fast Janus Green B would be a good stain to use on freehand sections.

138. **Metanil Yellow** is an acid dye, light 4 to 5. Cells of parenchyma are stained a fugitive yellow. Upon the addition of potassium dichromate the pigment becomes light brown.

142. **Methyl Orange,** Helianthine, Orange III is an acid dye, light 4. Because Methyl Orange is not affected by carbonates or weak organic acids it is used principally as an indicator. On paraffin sections it is similar to Orange G in both color and fastness.

143. **Orange IV,** Tropaeolin OO is an acid dye, light 4 to 5. Cells of parenchyma are yellow in acid solutions of the dye but

light brown in the presence of potassium dichromate. Without the addition of acids the dye has slight staining properties.

148. **Tropaeolin O** is an acid dye, light 3; ½ gram in 95 ml. of distilled water, 1 ml. of 1 per cent sulfuric acid, and 5 ml. of ½ per cent potassium dichromate. Vascular plant tissues are stained yellowish orange to brown of moderate fastness. Blue-green algae are clear yellow in alkaline solutions of the dye.

151. **Orange II,** Acid Orange II, Gold Orange, Tropaeolin OOO No. 2 is an acid dye, light 3 to 4; ½ gram in 100 ml. of distilled water and 1 ml. of 1 per cent sulfuric acid.

In staining vertebrate tissues or whole mounts of invertebrates use a mixture consisting of two parts Sun Yellow, C. I. No. 620, and one part Orange II for five to ten minutes and counterstain either in Aniline Blue, C. I. No. 707, for 15 seconds or in Niagara Sky Blue, C. I. No. 520, for 30 seconds. In a combination with Acid Fuchsine, C. I. No. 692, use nine parts of Orange II and one part Acid Fuchsine for two minutes and follow with Niagara Sky Blue for 30 seconds. If the tissue differentiates clearly, the red, orange, and blue provide a striking contrast.

On sections of animal or fungous tissues use a mixture of Sun Yellow and Orange II for 10 minutes and counterstain in Niagara Sky Blue. Aniline Blue may be tried as a counterstain either with Orange II alone or with the mixture of Sun Yellow and Orange II. A very good triple stain on plant tissues includes a mixture of two parts Orange II and one part Biebrich Scarlet for 24 hours. After a rinse in distilled water, counterstain meristems with Niagara Sky Blue; woody tissues may be counterstained in Niagara Sky Blue, Methyl Violet, C. I. No. 680, or New Methylene Blue, C. I. No. 927.

Treat mature plant tissues with ½ per cent potassium dichromate for 15 minutes, rinse in distilled water, and stain for 24 hours in Orange II. The counterstains are: (1) Aldehyde Green, C. I. No. 676a, five to ten minutes for blue-green and orange, (2) Thionine, C. I. No. 920, for blue and orange, (3) Victoria Blue B, C. I. No. 729, five minutes, (4) Methyl Violet one minute, or (5) New Methylene Blue one to five minutes.

Combinations of Stains

Double stains		*Counterstains*
Orange II	×	(1) Aldehyde Green
		(2) Aniline Blue
		(3) Methyl Violet
		(4) New Methylene Blue
		(5) Niagara Sky Blue
		(6) Thionine
		(7) Victoria Blue B
Triple stains		
Orange II (5) and		
Biebrich Scarlet (2)	×	(1) Methyl Violet
		(2) New Methylene Blue
		(3) Niagara Sky Blue
		(4) Victoria Blue B
Orange II (10) and		
Acid Fuchsine (1)	×	(1) New Methylene Blue
		(2) Niagara Sky Blue
Quadruple stains		
Sun Yellow (10),		
Orange II (5), and		
Crocein Scarlet MOO (5) or		
Biebrich Scarlet (2) or		
Acid Fuchsine (1) ...	×	(1) New Methylene Blue
		(2) Niagara Sky Blue
		(3) Aniline Blue

161. **Orange R** is an acid dye, light 3 to 4; ½ gram in 100 ml. of distilled water and 1 ml. of 1 per cent sulfuric acid. Stain sections of animal tissues in a mixture of two parts Sun Yellow, C. I. No. 620, and one part Orange R for ten minutes, rinse in distilled water, and counterstain in Erie Violet BW, C. I. No. 387, eight minutes for orange, red, and violet.

On sections of animal or fungous tissues use a mixture of Sun Yellow and Orange R for 10 minutes; counterstain in Niagara Sky Blue, C. I. No. 520, for one or two minutes. This combination stain does not give satisfactory colors on tissues taken from a fixative containing picric acid. On plant tissues, Orange R has a more reddish shade than Orange II; the counterstains are: (1) Methyl Violet, C. I. No. 680, (2) Aldehyde Green, C. I. No. 676a, (3)

Thionine, C. I. No. 920, (4) New Methylene Blue, C. I. No. 927, or (5) Victoria Blue B, C. I. No. 729.

168. **Superchrome Garnet Y** (NAC) is a mordant dye, light 3. Paraffin sections of plant tissues are stained orange-yellow to brown. The color becomes darker with an after-treatment in potassium dichromate. Blue-green algae are stained orange-red in an alkaline solution of the dye at 50° C. The pigment gradually disappears in glycerin.

169. **Superchrome Violet B** (NAC) is an acid mordant dye, light 3. Paraffin sections of plant tissues appear bright pink; they are more fast to washing with an after-treatment in 5 per cent ammonium alum. A mordant of potassium dichromate and acid potassium tartrate gives a reddish brown on parenchyma. Blue-green algae in an alkaline bath containing potassium dichromate, heated to 50° C., are dark carmine. The color disappears slowly in 10 per cent glycerin.

170. **Superchrome Black PV** (NAC) is an acid mordant dye. Paraffin sections of plant tissues stain a dull bordeaux; with a mordant, the color is bordeaux to brown. Blue-green algae in a hot alkaline bath are violet to blue-black; the pigment is not fast in glycerin.

175. **Naphthylamine Brown** is an acid dye, light 3 to 4. Plant tissues stain light brown to rich red-brown; in the presence of potassium dichromate they are darker brown.

176. **Fast Red A** is an acid dye, light 4. This stain does not differentiate well but blends with blue dyes.

179. **Azorubine** is an acid dye, light 4. Commercially, Azorubine is used as a substitute for the more expensive Acid Fuchsine. Plant tissues are stained brilliant red that contrasts well with blue dyes. Unfortunately, the color fades rapidly.

184. **Amaranth,** Naphthylamine Red is an acid dye, light 3 to 4. This dye is used principally as a food color.

185. **Brilliant Scarlet 3R** is an acid dye. Most materials are stained pink which is too pale to be useful. The stain becomes moldy on standing.

207. **Lanacyl Violet B** is an acid dye, light 3; ½ gram in 95 ml. of distilled water, 1 ml. of 1 per cent sulfuric acid, and 5 ml. of ½ per cent potassium dichromate. Parenchymatous tissue, cell walls, and fungous mycelia are stained clear blue-violet very similar in appearance to tissues taken from alum Haematoxylin.

210. **Lanacyl Blue BB** is an acid dye. Cell walls and parenchymatous cells are stained light blue. The dye is not so good as other blue acid dyes.

211. **Methyl Red** is used principally as an indicator. It has little value as a stain.

Class IV. Disazo Colouring Matters

248. **Sudan III** is unsuited for staining paraffin sections. Blue-green algae are stained a fugitive orange-red in an alkaline solution of the dye. As a fat stain this dye is used in the same manner as Sudan IV, C. I. No. 258.

252. **Crocein Scarlet MOO,** Carmoisine is an acid dye, light 3; ½ gram in 100 ml. of distilled water and 1 ml. of 1 per cent sulfuric acid. On animal tissues this stain is more valuable than Acid Fuchsine. Use Crocein Scarlet MOO for 10 minutes and counterstain in Niagara Sky Blue, C. I. No. 520, for one minute. With a mixture containing two parts Sun Yellow, C. I. No. 620, and one part Crocein Scarlet MOO for 10 minutes, the counterstains are: (1) Niagara Sky Blue one minute for red, green, and blue, (2) Aniline Blue, C. I. No. 707, which is not so selective as Niagara Sky Blue, 15 seconds, or (3) Erie Violet BW, C. I. No. 387, five minutes for red, violet, and green.

Sections of fungous tissues remain in Crocein Scarlet MOO for 10 minutes and are counterstained in Niagara Sky Blue one minute. Any of the combinations suggested for animal tissues should be tried on fungi. On vascular plants this bright red dye differentiates well with blue basic stains. It is similar to Azorubine in color and staining reactions but is more fast. It counterstains well with (1) Aldehyde Green, C. I. No. 676a, one to ten minutes, or (2) Opal Blue, C. I. No. 689, 30 minutes with a direct transfer from the stain to 95 per cent alcohol. Fairly good con-

trasts are obtained with Thionine, C. I. No. 920, Methylene Green, C. I. No. 924, or New Methylene Blue, C. I. No. 927.

258. **Sudan IV** is insoluble in water but soluble in alcohol, reddish violet in alkaline solutions; 1/10 gram in 50 ml. of 95 per cent alcohol and 50 ml. of 1 per cent sulfuric acid; heat to 50° C. From paraffin sections of various tissues the fats and oils are extracted with alcohol and xylene. Those structures which formerly contained fats are readily stained with either basic or direct dyes.

Freehand sections of fresh tissue are placed in Sudan IV at 50° C. for an hour or longer. The tissues containing fats become bright red which is slightly more intense and darker than the color produced by Sudan II, C. I. No. 73, or Sudan III, C. I. No. 248. Tissues consisting of carbohydrates or proteins remain unchanged.

266. **Janus Red B** is a basic dye, light 4. Parenchyma becomes fugitive pink to red depending on the length of time in the stain.

280. **Biebrich Scarlet,** Crocein Scarlet 5R is an acid dye, light 3; ½ gram in 100 ml. of distilled water and 1 ml. of 1 per cent sulfuric acid. This is the best red acid dye for staining plant tissues. It is more scarlet and more fast than Azorubine; it may be used in techniques calling for Acid Fuchsine. Tissues fixed in formalin, picric-formalin-acetic, or in Zenker's solution take a brilliant color; after Gilson's fixative the stain is not always satisfactory. As a rule Biebrich Scarlet does not overstain mature tissues but differentiates easily with green, blue, or violet basic dyes.

Use Biebrich Scarlet on vascular tissues 24 hours and counterstain with: (1) Methyl Violet, C. I. No. 680, one to three minutes, (2) Victoria Blue B, C. I. No. 729, five minutes, (3) New Methylene Blue, C. I. No. 927, five minutes, or (4) Aldehyde Green, C. I. No. 676a, five minutes for a mixture of red, green, and greenish black.

Except for certain invertebrates Biebrich Scarlet is not so good on animal tissues. On whole mounts of small animals use a mixture of ten parts Sun Yellow, C. I. No. 620, and two parts Biebrich Scarlet for five minutes; follow with Niagara Sky Blue, C. I. No. 520, for 15 to 30 seconds.

Combinations of Stains

Double stains		*Counterstains*
Biebrich Scarlet	×	(1) Aldehyde Green (2) Aniline Blue (3) Methyl Violet (4) New Methylene Blue (5) Niagara Sky Blue (6) Victoria Blue B
Triple stains		
Biebrich Scarlet (2) and Orange II (5)	×	(1) Methyl Violet (2) New Methylene Blue (3) Niagara Sky Blue
Biebrich Scarlet (2) and Sun Yellow (10)	×	(1) New Methylene Blue (2) Niagara Sky Blue
Quadruple stains		
Biebrich Scarlet (2), Orange II (5), and Sun Yellow (10)	×	(1) New Methylene Blue (2) Niagara Sky Blue

286. **Crocein Scarlet 7B** is an acid dye, light 3; ½ gram in 100 ml. of distilled water and 1 ml. of 1 per cent sulfuric acid. This stain has properties similar to those of Biebrich Scarlet except that it is more fuchsia. It may be used on animal tissues instead of Acid Fuchsine. On sections of animal tissues use one of the triple stains. Place the material in a mixture of five parts Sun Yellow, C. I. No. 620, and one part Crocein Scarlet 7B for five minutes; the counterstains are: (1) Niagara Sky Blue, C. I. No. 520, one minute, (2) Aniline Blue, C. I. No. 707, 30 seconds, (3) Direct Green B, C. I. No. 593, two minutes, or (4) Erie Violet BW, C. I. No. 387, eight minutes.

On mature plant tissues use Crocein Scarlet 7B for 24 hours and counterstain with (1) Methyl Violet, C. I. No. 680, three minutes, (2) Victoria Blue B, C. I. No. 729, ten minutes, (3) Niagara Sky Blue five minutes, or (4) Aniline Blue five minutes.

289. **Tolyl Blue 5R** is a mordant dye, light 3; ½ gram in 100 ml. of 1 per cent ammonium acetate. With potassium dichromate as the mordant, tracheids, pollen, and fungous mycelia are blue in

the stain containing acetic acid. These results indicate that the dye is relatively inactive at room temperature. If the material is mordanted in ferric ammonium sulfate and stained for three hours at 50° C., meristems are clear blue; brown and red algae are dark blue. Nuclei become darker than the cytoplasm. In alkaline solutions of the dye, blue-green algae stain a clear brilliant blue but the color disappears in glycerin.

Sections of animal tissues are placed in the mordant 1 per cent ferric alum for 15 minutes. After a rinse in distilled water, Tolyl Blue 5R stains the material at 50° C. within five to ten minutes. The sections are clear bright blue.

315. **Wool Black B** is an acid dye, light 3 to 4. Parenchyma stains a clear blue; sclerenchyma is tinted green to dull greenish black. In the presence of potassium dichromate, nuclei remain uncolored but the cytoplasm stains blue-green.

326. **Direct Fast Orange,** Erie Fast Orange (NAC), Erie Fast Scarlet YA (NAC) is a direct dye, light 3.

327. **Direct Fast Scarlet 4BS,** Pontamine Fast Scarlet 4BS (Du Pont) is a direct dye, light 3; ½ gram in 95 ml. of distilled water, 5 ml. of 5 per cent sodium sulfate, and ½ ml. of acetic acid. Direct Fast Orange and Direct Fast Scarlet differ only in color. The two dyes are not so good on tissues taken from Gilson's or Bouin's fixative. Whenever alcohol, formalin, and acetic acid are used in one of the many modifications of fixatives, animal tissues become brilliant orange-red in 15 to 30 minutes, but mature plant tissues need an overnight stain for a full development of the color. Fortunately, the dye does not overstain. It is fast to washing in water, alcohol, ferric alum, and remarkably fast to acids, including *nitric acid.*

Direct Fast Scarlet 4BS may be used as a general stain instead of Carmine on paraffin sections of animal or fungous tissues. The dye is most useful as a counterstain. The material is first stained in iron Haematoxylin, differentiated in iron alum, and then counterstained in Direct Fast Scarlet 4BS—animal tissues 15 to 30 minutes, meristematic plant tissues 30 minutes, mature plant tissues overnight. The differential staining of parasitic fungi and

plant host should be tried with alum Haematoxylin and Direct Fast Scarlet 4BS. This latter combination of stains provides the contrast which has been attempted so often with Haematoxylin and Congo Red. Should Direct Fast Scarlet 4BS be the first stain used on mature plant tissues counterstain the material with Methyl Violet, C. I. No. 680.

331. **Bismarck Brown G,** Y, and 332. **Bismarck Brown R,** Vesuvine R are basic dyes, light 3; ½ gram in 95 ml. of distilled water, 5 ml. of 5 per cent ammonium alum, and ½ ml. of acetic acid. An overnight stain on plant tissues gives an intense brown; the R shade is the darker. On paraffin sections, Bismarck Brown is not equal to Acridine Orange in fastness.

353. **Pontamine Fast Pink BL** (Du Pont) is a direct dye, light 3 to 4. A fuchsia pigment is obtained on paraffin sections of various plant tissues with an acid solution of the dye. With a potassium dichromate mordant, parenchyma is stained brick red. In alkaline solutions of the dye, blue-green algae are colored a fugitive pink.

364. **Brilliant Yellow** is an acid dye, light 3. For most sections of materials embedded in paraffin the color is too light; resinous tissues stain bright yellow from an acid solution. Blue-green algae are a clear yellow in alkaline solutions.

365. **Chrysophenine** is a direct dye, light 4 to 5. This dye is used as a substantive yellow on fabrics for bronze shades. On paraffin sections the stain is too light and too fugitive.

370. **Congo Red** is a direct dye, light 4; ½ gram in 95 ml. of distilled water and 5 ml. of 5 per cent sodium sulfate. On sections of animal tissues Congo Red is used as a contrast stain with Haematoxylin. The color is not uniformly distributed on most objects because the stain does not have a specific selection for any particular tissue.

If Congo Red were more fast to light it would be very useful for staining fungous mycelia within a plant host. With materials fixed in Gilson's solution the host appears purple in alum Haematoxylin; after the section is rinsed in distilled water, the fungous parasite is stained in Congo Red for 24 hours. The dye is so

soluble in water that the sections are dehydrated as quickly as possible after a direct transfer from the stain to 95 per cent alcohol. The brick red of stained fungi changes to dull brown if sections are left too long in xylene before mounting. Acids precipitate this dye from solution and change the color from red to blue; for that reason Congo Red is sometimes used as an indicator. Instead of Congo Red use Direct Fast Scarlet 4BS on either animal or plant tissues.

375. **Congo Corinth G,** Erie Garnet B is a direct dye, light 4. This dye is more orange and more fugitive than Congo Red.

387. **Erie Violet BW** (NAC) is an acid dye, light 2 to 3; ½ gram in 100 ml. of distilled water and 1 ml. of 1 per cent sulfuric acid. In sections of animal tissues Erie Violet BW may be used as a counterstain with either Crocein Scarlet MOO, C. I. No. 252, or Crocein Scarlet 7B, C. I. No. 286.

On plant tissues the cytoplasm of meristematic cells is stained violet while the nuclei remain uncolored. Storage parenchyma and cells containing resin are colored reddish violet to violet-black.

394. **Erie Violet 3R** (NAC) is a direct dye, light 3. This dye is not so good as Erie Violet BW, C. I. No. 387, for staining either animal or plant materials. Parenchyma is generally red to reddish violet with the cell walls violet.

410. **Chrysamine G** is an acid dye, light 5. Stained tissues are too fugitive to be of any value.

415. **Pontachrome Orange R** (Du Pont) is a direct dye, light 5. A neutral stain gives a fugitive orange on paraffin sections of plant tissues. In an alkaline solution, blue-green algae are stained a fugitive bright orange.

419. **Erie Fast Red FD** (NAC) is a direct dye, light 3 to 4. All of the color is removed from stained paraffin sections upon washing in water or in 95 per cent alcohol; in this respect the dye resembles Congo Red. In alkaline solutions blue-green algae are deep red to reddish brown.

438. **Trypan Red** is a direct dye, light 4. A bright red is obtained on plant tissues but the color is not very fast. Trypan Red may be used as a vital stain.

448. **Benzopurpurin 4B** is a direct dye, light 4 to 5. This dye is less intense and more fugitive than Congo Red. It may be used in vital staining.

450. **Benzopurpurin B** is a direct dye, light 4. Parenchyma is stained a bright fugitive red.

463. **Azo Blue** is a direct dye, light 4. Azo Blue was the first blue azo dye known; it was seldom used because of the ugly reddish shade produced on fabrics. For paraffin sections the color is too fugitive. It is polychromatic, staining nuclei red and cytoplasm blue. For polychromatic staining with a direct blue dye use Niagara Sky Blue, C. I. No. 520.

477. **Diamine Blue 3B,** Trypan Blue is a direct dye, light 4. Parenchyma is stained a bright blue; nuclei are sometimes tinted red. It is used as a vital stain.

495. **Benzopurpurin 10B** is a direct dye, light 4 to 5. The bright red on parenchyma is similar to the color produced by Congo Red.

502. **Benzoazurine G** is a direct dye, light 4. Sometimes the stain is polychromatic; then, the nuclei of parenchyma retain red; the cytoplasm, blue to blue-violet. In green algae previously treated with copper sulfate, spores are tinted orange-red; the vegetative cells are dark blue or violet.

518. **Niagara Sky Blue 6B** is a direct dye, light 3; ½ gram in 95 ml. of distilled water, 5 ml. of 5 per cent sodium sulfate, and ½ ml. of acetic acid. The two best direct blue dyes are Niagara Sky Blue 6B and Niagara Sky Blue, C. I. No. 520. The former dye cannot be used on tissues fixed with Gilson's solution, the latter can. There is little difference in the appearance of the dye powders or the solutions but the results obtained from the two stains are unlike. The first dye is a shade lighter blue, requires two or three times as long for staining, and, in combinations with Sun Yellow, C. I. No. 620, gives a clear brilliant green. For a contrast in green and red, with tints of yellow and blue, use a mixture of nine parts Sun Yellow and one part Acid Fuchsine, on sections of animal or fungous tissue for 10 to 30 minutes, and counterstain in Niagara Sky Blue 6B, two or three minutes. Niagara Sky Blue 6B should be tried

on either animal or plant tissues using the combinations of stains given under Niagara Sky Blue, C. I. No. 520.

520. **Niagara Sky Blue,** Direct Sky Blue is a direct dye, light 3; ½ gram in 95 ml. of distilled water, 5 ml. of 5 per cent sodium sulfate, and ½ ml. of acetic acid. Niagara Blue 4B is a commercial trade name originating from a former manufacturer of dyes. Frequently it is necessary to employ a dye possessing basic properties, the $-NH_2$ radical, in order to produce a contrast with acid dyes. Niagara Sky Blue supplies this need in many sections of animal or fungous tissues where the structures are differentiated more clearly than with Aniline Blue, C. I. No. 707. Suggestions for the application of this blue counterstain are given under Sun Yellow, C. I. No. 620, Orange II, C. I. No. 151, Crocein Scarlet MOO, C. I. No. 252, Biebrich Scarlet, C. I. No. 280, and Acid Fuchsine, C. I. No. 692.

On red and green algae, the polychromatic properties are evident; the spores are tinted red and the vegetative cells, blue to violet. On mature plant tissues, the basic dyes such as Rhodamine 6G, C. I. No. 752, Acridine Red, C. I. No. 740, or Safranine, C. I. No. 841, are followed by Niagara Sky Blue for one to five minutes. Should the section consist largely of parenchyma, Biebrich Scarlet and Niagara Sky Blue often supply a vivid contrast. On either fungous tissues or meristems, the orange and red acid dyes such as Orange II, Biebrich Scarlet, or Acid Fuchsine used for 30 minutes to 24 hours may be followed by Niagara Sky Blue one to five minutes in the same manner as these combinations are used on animal materials.

Class V. Trisazo Colouring Matters

581. **Erie Black GXOO,** Chlorazol Black E, Direct Black E is a direct dye. In the commercial dyeing of cotton, Erie Black is applied at boiling temperature with the aid of a chromium salt. In sections of embryos taken from a fixative containing chromium as in chrom-acetic or Zenker's solution, the dye colors the nuclei black and cytoplasm gray. The dye cannot be used as a general stain on either animal or plant tissues. Parts of a pine needle section are pale blue, dingy green, or dim yellow; on invertebrate and verte-

brate tissues the unevenly distributed dull black is undesirable. Specialized uses of Erie Black do not compare favorably with Niagara Sky Blue, C. I. No. 520, or Haematoxylin, C. I. No. 1246.

593. **Direct Green B,** Diazine Green B is a direct dye, light 3 to 4. There are few green acid dyes suitable for animal tissues. On invertebrates or paraffin sections, try Crocein Scarlet 7B, C. I. No. 286, for five minutes and counterstain with Direct Green B two minutes.

The spores of blue-green algae are blue-green or black; the vegetative cells, clear blue. In an acid solution of the dye, green or red algae are given a polychromatic stain that varies through red to purple or blue. Parenchyma of vascular plants is dull blue.

594. **Direct Green G,** Alkali Green D is a direct dye, light 4 to 3; ½ gram in 95 ml. of distilled water, 5 ml. of 5 per cent sodium sulfate, and 1 ml. of 4 per cent sodium hydroxide. This formula constitutes the stain for blue-green algae and whole mounts giving the best direct green; it is moderately fast in glycerin. On paraffin sections of animal tissues and vascular plants the dark green is too indefinite.

Class VI. Tetrakisazo Colouring Matters

This class includes yellow, brown, and black direct dyes that are not used as biological stains.

Chapter VI

BIOLOGICAL STAINS

A. Synthetic Organic Dyes (*Continued*)
Classes VII to XXVI

Class VII. Stilbene Colouring Matters

620. **Sun Yellow** is a direct dye, light 3; ½ gram in 95 ml. of distilled water, 5 ml. of 5 per cent sodium sulfate, and ½ ml. of acetic acid. A few direct dyes react in a manner similar to a mordant; such dyes are not always evident in the finished slide but they assist in the fixation of the more important dye. Because the color of Sun Yellow in combinations with blue dyes changes to green, the relative amounts of yellow, green, and blue can be used as an indicator to show when the proper degree of contrast has been obtained. This dye on both animal and plant materials serves as a mordant in the production of green in combinations with blue counterstains; this green is more fast to light than Fast Green FCF, C. I. No. 670.

On whole mounts of invertebrates or on paraffin sections of tissues, use Sun Yellow ten minutes; in mixtures with Orange II, C. I. No. 151, the Crocein Scarlets, or Biebrich Scarlet, C. I. No. 280, for five minutes; in mixtures with Acid Fuchsine, C. I. No. 692, try one to five minutes. The contrast dyes include: (1) Aniline Blue, C. I. No. 707, 15 to 30 seconds, (2) Niagara Sky Blue, C. I. No. 520, 30 seconds to one minute, (3) New Methylene Blue, C. I. No. 927, one minute, or (4) Erie Violet BW, C. I. No. 387, five to eight minutes.

Use Sun Yellow or the mixtures containing Sun Yellow for 24 hours on mature plant tissues but ten minutes to an hour on meristems. The counterstains require 15 seconds to five minutes depending on the time given to the lighter stain.

Combinations of Stains

Double stains		*Counterstains*
Sun Yellow	×	(1) Aniline Blue (2) New Methylene Blue (3) Niagara Sky Blue
Triple stains		
Sun Yellow (10) and Orange II (5)	×	(1) New Methylene Blue (2) Niagara Sky Blue
Sun Yellow (10) and Crocein Scarlet MOO (5)	×	(1) Aniline Blue (2) New Methylene Blue (3) Niagara Sky Blue
Sun Yellow (10) and Biebrich Scarlet (2)	×	(1) New Methylene Blue (2) Niagara Sky Blue
Sun Yellow (10) and Crocein Scarlet 7B (2) ..	×	(1) Aniline Blue (2) New Methylene Blue (3) Niagara Sky Blue (4) Direct Green B (5) Erie Violet BW
Sun Yellow (10) and Acid Fuchsine (1)	×	(1) Aniline Blue (2) New Methylene Blue (3) Niagara Sky Blue (4) Niagara Sky Blue 6B
Quadruple stains		
Sun Yellow (10), Orange II (5), and Crocein Scarlet MOO (5)	×	(1) New Methylene Blue (2) Niagara Sky Blue
Sun Yellow (10), Orange II (5), and Biebrich Scarlet (2)	×	(1) Aniline Blue (2) New Methylene Blue (3) Niagara Sky Blue
Sun Yellow (10), Orange II (5), and Acid Fuchsine (1)	×	(1) Aniline Blue (2) New Methylene Blue (3) Niagara Sky Blue

622. **Mikado Yellow G,** Stilbene Yellow is a direct dye, light 3. The stain is lighter than Sun Yellow but it is similar in other respects.

Class VIII. Pyrazolone Colouring Matters

640. **Tartrazine** is an acid dye, light 4. The bright yellow dye is valued for the coloring of foodstuffs, for light filters, or for orthochromatic photographic plates.

Class IX. Ketonimine Colouring Matters

This class includes **Auramine,** C. I. No. 655, a basic dye that is too fugitive for use on permanent slides.

Class X. Triphenylmethane and Diphenylnaphthylmethane Colouring Matters

Although the first basic dyes were made from aniline, very few of the coal-tar dyes originate from that source, either directly or indirectly. Because the early basic dyes were fugitive there arose a popular impression that all synthetic dyes were likewise deficient in fastness. Many of these dyes exhibit the phenomenon of surface color to a marked degree. Basic Fuchsine, C. I. No. 677, is red when dissolved in water; the crystals have a beautiful beetle green surface which is complementary in color to that of the solution. Because the basic stains in pure water readily undergo a partial hydrolysis of the dye salt, they are dissolved in distilled water with acetic acid. On exposure to sunlight, all green basic dyes fade rapidly; this is attributed partly to the greater absorption of light rays by green pigments.

657. **Malachite Green,** China Green, Diamond Green, Solid Green is a basic dye, light 4; ½ gram in 95 ml. of distilled water, 5 ml. of 5 per cent ammonium alum, and ½ ml. of acetic acid. Malacite Green is frequently used on xylem and sclerenchyma where it is better than Methyl Green, C. I. No. 684. For a contrast of red and green, stain the sections of plant tissues first in Crocein Scarlet MOO, C. I. No. 252, Biebrich Scarlet, C. I. No. 280, or Acid Fuchsine, C. I. No. 692, and follow with Malachite Green for 12 to 15 minutes.

658. **Setoglaucine O** is a basic dye, light 5. The dye is lighter and less fast than Malachite Green, C. I. No. 657. It is used in certain bacteriological media.

662. **Brilliant Green,** Ethyl Green is a basic dye, light 5. This dye has the same staining properties as Setoglaucine O, C. I. No. 658. It, too, is used in bacteriological media.

665. **Alkali Green** is an acid dye, light 5. The pale dull green obtained with Alkali Green is one of the most fugitive.

670. **Light Green SF,** Acid Green G, Fast Green FCF is an acid dye; light 5; ½ gram in 100 ml. of distilled water and 1 ml. of 1 per cent sulfuric acid. Plant tissues are stained a fugitive green or light blue-green. Fast Green FCF, which is the darker, should be used only on thick sections of vascular tissues. Mature plant materials taken from one of the basic dyes, Rhodamine, C. I. No. 752, Basic Fuchsine, C. I. No. 677, or Safranine, C. I. No. 841, are counterstained in Fast Green FCF for 12 to 15 minutes. More useful green cytoplasmic stains that have been described are the combinations of Sun Yellow, C. I. No. 620, with New Methylene Blue, Aniline Blue, or Niagara Sky Blue. On invertebrates and whole mounts of fungi or algae, Naphthol Green B, C. I. No. 5, applied with the aid of ferric alum produces a green more fast and better differentiated than Fast Green FCF. Naphthol Green B is also the better contrast green after iron Haematoxylin.

A quadruple stain which includes Fast Green FCF may be applied to vascular tissues. The first stain consisting of a mixture of five parts Orange II and one part Fast Green FCF is used for 15 to 30 minutes. After a rinse in distilled water, the material is counterstained in three parts Safranine and one part Methyl Violet for 15 to 30 seconds. If more of the green is wanted, use the first mixed stain longer than 30 minutes; with a longer interval in Safranine and Methyl Violet, the percentage of violet is increased.

676. **Pararosaniline** is a basic dye, light 2; 1/10 gram in either 95 ml. of distilled water or 95 ml. of 50 per cent alcohol, 5 ml. of 5 per cent ammonium alum, and ½ ml. of acetic acid. The fuchsia on plant tissues is brighter than the bluish red obtained with Basic Fuchsine, C. I. No. 677. The dye may be used in staining bacteria or in formulae calling for Basic Fuchsine.

676a. **Aldehyde Green,** Aniline Green, Benzaldehyde Green is a basic dye, light 4; ½ gram in 95 ml. of distilled water, 5 ml. of 5 per cent ammonium alum, and ½ ml. of acetic acid. As a counterstain, use Aldehyde Green for 15 minutes after Biebrich Scarlet, C. I. No. 280, Acid Fuchsine, C. I. No. 692, Crocein Scarlet MOO, C. I. No. 252, or Orange II, C. I. No. 151. It is similar to Malachite Green, C. I. No. 657, but on xylem and sclerenchyma the green is a shade brighter.

677. **Basic Fuchsine,** Aniline Red, Diamond Fuchsine, Magenta, Roseine is a basic dye, light 2; ½ gram in 95 ml. of distilled water, 5 ml. of 5 per cent ammonium alum, and ½ ml. of acetic acid; warm the mixture until the dye dissolves. Although Basic Fuchsine is more or less fugitive on fabrics, it is comparatively fast on paraffin sections. It is used in certain bacteriological media and in the staining of bacteria. Vascular plant tissues are stained an intense fuchsia to reddish violet in 15 minutes; the best counterstain is Fast Green FCF, C. I. No. 670. For a good triple stain on free-hand sections use a mixture of nine parts Orange II, C. I. No. 151, and one part Benzo Pure Blue, C. I. No. 520, for one hour, and after a rinse in distilled water, counterstain with Basic Fuchsine one minute.

678. **New Fuchsine,** New Magenta is a dye similar to but more soluble and more bluish red than Basic Fuchsine.

679. **Dahlia,** Hoffmann Violet, Iodine Violet, Red Violet 5R is a basic dye, light 2 to 3; 1/10 gram in 95 ml. of distilled water, 5 ml. of 5 per cent ammonium alum, and ½ ml. of acetic acid. The dye may be used in any of the procedures described under Methyl Violet, C. I. No. 680. A fast pigment is obtained if the material is stained for an hour and differentiated in 95 per cent alcohol. Depending on the tissue, the color varies from a reddish violet to dark violet or black.

680. **Methyl Violet B,** 2B is a basic dye, light 2 to 3; 1/10 gram in 95 ml. of distilled water, 5 ml. of 5 per cent ammonium alum, and ½ ml. of acetic acid. On animal tissues, basic dyes have a limited value; nuclei and a few kinds of special glandular cells such as goblet cells of the intestine and mucous cells of the epidermis are colored much darker than the nonspecialized cytoplasmic structures.

When compared with other shades of Methyl Violet on the same kind of plant tissues, the 2B shade is the best of the series. As a counterstain after Acid Fuchsine, C. I. No. 692, Biebrich Scarlet, C I. No. 280, Crocein Scarlet MOO, C. I. No. 252, or Orange II, C. I. No. 151, Methyl Violet B may be used for from five seconds to five minutes depending on the time taken for the first and lighter color. An especially noteworthy triple stain for root, stem, and leaf sections is obtained by using a mixture of two parts Orange II and one part Biebrich Scarlet for 24 hours, and after a rinse in distilled water, a counterstain in Methyl Violet B for two or three minutes. The colors are very fast as well as brilliant.

681. **Crystal Violet,** Methyl Violet 10B is a basic dye, light 3. Gentian Violet is either a mixture of two or more shades of Methyl Violet or it is Crystal Violet containing dextrin. Crystal Violet is more selective in its staining of minute cell structures than other basic violet dyes. The dye is dissolved in distilled water with ammonium alum and acetic acid; it is used in the same manner as and after the same acid dyes given in combinations with Methyl Violet B.

682. **Ethyl Violet** is a basic dye, light 5. Tissues are not stained uniformly and the color is very fugitive.

683. **Methyl Violet 6B,** Benzyl Violet, Gentian Violet 6B is a basic dye, light 3; 1/10 gram in 95 ml. of distilled water, 5 ml. of 5 per cent ammonium alum, and $\frac{1}{2}$ ml. of acetic acid. Plant tissues are stained blue-violet similar to the color obtained with Crystal Violet but the differentiation of minute cell structures is not so clear. As a counterstain, Methyl Violet 6B is used for three minutes after most of the red acid dyes such as Acid Fuchsine, Crocein Scarlet MOO, or Orange II.

684. **Methyl Green** is a basic dye, light 4. The Methyl Green now on the market, both the domestic and imported product, is a blue or blue-green dye; it is unstable and fugitive. The green dye of former manufacture, labeled Methyl Green, was a different dye or a mixture. On various plant tissues the stain is blue-green to blue-violet. Instead of Methyl Green use Methylene Green, C. I. No. 924.

686. **Iodine Green** is a basic dye, light 4. Different plant tissues are stained blue-green or dull blue without a trace of green. Similar to Methyl Green, it should be replaced by the better dye, Methylene Green, C. I. No. 924.

689. **Opal Blue,** Aniline Blue alcohol soluble, Bleu Lumiere, Gentiana Blue 6B, Spirit Blue is a basic dye, light 3; ½ gram in 95 ml. of 50 per cent alcohol, 5 ml. of 5 per cent ammonium alum, and ½ ml. of acetic acid. The cytoplasm is more readily stained an intense blue than are the nuclei. Opal Blue may be used whenever the color produced by New Methylene Blue is too dark. In contrast staining use Biebrich Scarlet, C. I. No. 280, Crocein Scarlet MOO, C. I. No. 252, or Orange II, C. I. No. 151, for 24 hours and Opal Blue five minutes; transfer the section from the stain directly to 95 per cent alcohol for dehydration.

692. **Acid Fuchsine,** Acid Azo Fuchsine, Acid Magenta, Acid Rubine, Fuchsine S, Rubine S is an acid dye, light 2 to 3; ½ gram in 100 ml. of distilled water and 1 ml. of 1 per cent sulfuric acid. The dye sold previous to 1920 was a fugitive red dye, probably Azorubine; that now on the market, domestic or imported, if true to name, gives a fuchsia to reddish violet of good fastness. By the sulfonation of different Basic Fuchsines, various quantities of the derived acid dye are red to violet depending on the particular basic dye used in its manufacture. If a bright red is not obtained with Acid Fuchsine, use Biebrich Scarlet, C. I. No. 280, on plant tissues or Crocein Scarlet MOO, C. I. No. 252, on animal tissues.

In the staining of animal tissues, Acid Fuchsine usually blends with blue or violet dyes to give colors of little contrast. If Acid Fuchsine is diluted with either Sun Yellow, C. I. No. 620, or Orange II, C. I. No. 151, the contrast with blue stains will be brilliant. A good combination of stains for whole mounts, invertebrates, chick embryos, or paraffin sections of tissues includes a mixture of ten parts Sun Yellow and one part Acid Fuchsine for five minutes and a counterstain either in Aniline Blue, C. I. No. 707, 15 seconds, or in Niagara Sky Blue, C. I. No. 520, 30 seconds. Another good technique includes a mixture of ten parts Orange II and one part Acid Fuchsine for one or two minutes and a counterstain in Aniline Blue or Niagara Sky Blue.

In staining mosses, ferns, or seed plants, use a mixture of twenty parts Sun Yellow and one part Acid Fuchsine for 24 hours; counterstain in New Methylene Blue, C. I. No. 927, five minutes. The basic dye adds a clear outline to the cell walls. Because fungous tissues are similar to animal tissues in their reactions to dyes, the combinations given in the previous paragraph should be used. Double stains on vascular plants require Acid Fuchsine for 15 minutes or longer and a counterstain: (1) Aldehyde Green, C. I. No. 676a, 15 minutes, (2) Methyl Violet B, C. I. No. 680, one to three minutes, (3) Methylene Green, C. I. No. 924, 15 minutes, (4) Victoria Blue B, C. I. No. 729, five minutes, (5) Thionine, C. I. No. 920, five minutes, or (6) Opal Blue, C. I. No. 689, one to ten minutes.

698. **Acid Violet** is an acid dye, light 4. The dye sold under this name is of variable composition. Because plant tissues are not stained uniformly, a good acid violet may be obtained with Erie Violet BW, C. I. No. 387; sometimes Acid Fuchsine, C. I. No. 692, is satisfactory.

703. **Alkali Blue 6B** is an acid dye, light 4 to 5. On parenchyma a fugitive blue to blue-green provides little of value for permanent slides. In alkaline solutions of the dye, blue-green algae become clear bright blue, but, unfortunately, the color is not fast.

705, 706, and 707. **Aniline Blue** water soluble, China Blue, Cotton Blue, Methyl Blue, Soluble Blue, Water Blue are closely related acid dyes, light 3; ½ gram in 95 ml. of distilled water, 5 ml. of 5 per cent ammonium alum, and 1 ml. of 1 per cent sulfuric acid.

On animal tissues, Aniline Blue and Niagara Sky Blue, C. I. No. 520, comprise an important pair of contrast stains. Should the former of these blue dyes not provide a clear differentiation of tissues, the latter usually supplies the desired quality. In most combinations of stains including Orange G, C. I. No. 27, Orange II, C. I. No. 151, Orange R, C. I. No. 161, Crocein Scarlet MOO, C. I. No. 252, or Sun Yellow, C. I. No. 620, Aniline Blue requires 15 seconds to one minute. Aniline Blue is also a constituent in several triple stains which are described under Sun Yellow, Orange II, and Crocein Scarlet MOO.

Aniline Blue is of greater importance on plant tissues than on animals. After a 24-hour stain in a basic dye such as Rhodamine 6G, C. I. No. 752, Acridine Red, C. I. No. 740, or Safranine, C. I. No. 841, the best blue counterstain is Aniline Blue for five minutes.

710. **Isamine Blue 6B** is an acid dye, light 3 to 4. The color and staining properties of the dye are similar to those of Aniline Blue; it is less fast.

712. **Acid Blue G,** Brilliant Acid Blue V is an acid dye, light 5. Parenchyma is stained a light blue-green that is lacking in definition of details and in fastness.

714. **Patent Blue A,** Brilliant Acid Blue A is an acid dye, light 4. Parenchyma is stained blue-green but the details of structure are not presented clearly.

720. **Eriochrome Azurol V** is a mordant dye, light 4 to 3; ½ gram in 90 ml. of distilled water, 5 ml. of ½ per cent potassium dichromate, and 5 ml. of 5 per cent sodium sulfate. Cell walls of parenchyma are stained a clear blue and the cytoplasm, violet; the nuclei remain unchanged. The color is similar to that obtained with Niagara Sky Blue.

724. **Aurin** is the free acid Rosolic Acid used as an indicator. Coralline, the sodium salt of Rosolic Acid, is used for the manufacture of turkey red lakes.

729. **Victoria Blue B** is a basic dye, light 3; ½ gram in 95 ml. of distilled water, 5 ml. of 5 per cent ammonium alum, and ½ ml. of acetic acid. A clear blue is obtained in parenchyma or sclerenchyma. Sometimes this stain gives better contrasts with red acid dyes than New Methylene Blue, C. I. No. 927. As a counterstain use Victoria Blue B after Acid Fuchsine, C. I. No. 692, Biebrich Scarlet, C. I. No. 280, Crocein Scarlet MOO, C. I. No. 252, or Orange II, C. I. No. 151.

731. **Night Blue** is a basic dye, light 4. The beautiful blue-violet obtained on plant tissues is not very fast to light.

736. **Acid Blue B** is an acid dye, light 5. Tissues stained with this dye are not clearly differentiated; they are too light and very fugitive.

Class XI. Xanthene Colouring Matters

The majority of the Xanthene dyes are characterized by the strong fluorescence of their solution which is attributed to the presence of the pyrone ring in the dye molecule.

739. **Pyronine 2G** is a basic dye, light 1 to 2; ½ gram in 95 ml. of distilled water, 5 ml. of 5 per cent ammonium alum, and ½ ml. of acetic acid. Plant tissues are stained a bright red to fuchsia. The color is similar to that of Basic Fuchsine, C. I. No. 677, but Pyronine 2G is more easily differentiated in double staining than Basic Fuchsine. The best contrast is obtained with either Niagara Sky Blue, C. I. No. 520, or alum Haematoxylin. For staining yeast spores or bacteria use 5 per cent of the dye instead of the ½ gram in the regular formula. A film of yeast containing spores is dried on a slide, left in 5 per cent chromic acid for 30 minutes, rinsed with distilled water, stained in Pyronine 2G at 50° C. for one hour, and counterstained in Niagara Sky Blue for five minutes.

740. **Acridine Red** is a basic dye, light 2; ½ gram in 95 ml. of distilled water, 5 ml. of 5 per cent ammonium alum, and ½ ml. of acetic acid. Plant tissues are pink to bright red. It may be used in any technique where Safranine, C. I. No. 841, gives good results but the color will be brighter than that of Safranine. A section taken from this stain may be counterstained in Aniline Blue, C. I. No. 707, Niagara Sky Blue, C. I. No. 520, or alum Haematoxylin, C. I. No. 1246.

741. **Pyronine B** is a basic dye with properties similar to those of Pyronine 2G; it is more bluish red.

749. **Rhodamine B,** 750. **Rhodamine G,** and 752. **Rhodamine 6G** are basic dyes, light 2; ½ gram in 95 ml. of distilled water, 5 ml. of 5 per cent ammonium sulfate, and ½ ml. of acetic acid. Rhodamine 6G is a bright pink; it is more easily differentiated with blue acid or direct dyes than any other red basic stain. Rhodamine 6G for 12 to 24 hours does not overstain paraffin sections, freehand sections of vascular plants, or woody materials. The counterstains include: (1) Aniline Blue, C. I. No. 707, five minutes, (2) Niagara Sky Blue, C. I. No. 520, five minutes for red and light blue, or (3) alum Haematoxylin, C. I. No. 1246, for bright red and purple.

764. **Phenolphthalein** is used extensively as an indicator.

766. **Fluorescein,** Uranine is an acid dye, light 5 to 4. Fluorescein does not stain plant tissues unless potassium dichromate is present, whereupon, a yellow to brown is obtained which is equal to Eosin in fastness.

768. **Eosin Y,** 769. **Methyl Eosin,** 770. **Ethyl Eosin,** and 771. **Eosin B** are acid dyes, light 4 to 5. Few dyes are so easily precipitated by acids as Eosin and Erythrosin. They are frequently used in counterstaining bacteria, fungi, animal tissues, or whole mounts. The fugitive nature of Eosin does not recommend its use for permanent preparations. By adding potassium dichromate to the stain, the fastness is improved slightly and the color is changed to a darker tint.

772. **Erythrosin G, Y,** and 773. **Erythrosin B** are acid dyes, light 4 to 5; ½ gram in 100 ml. of distilled water with or without the addition of 5 ml. of ½ per cent potassium dichromate. Potassium dichromate increases the fastness and changes the color to bright scarlet. Erythrosin is used as a food color. On animal tissues Erythrosin usually blends with other dyes to yield colors of little contrast. A fairly good differentiation occurs with a nuclear stain in Haematoxylin followed by Erythrosin or ten minutes in Erythrosin followed by Niagara Sky Blue, C. I. No. 520, for two minutes. Instead of Erythrosin, try Crocein Scarlet MOO, C. I. No. 252.

Sections of plant tissues are stained one to ten hours in Erythrosin and then counterstained with Capri Blue, C. I. No. 876, about five minutes, for pink and blue. This combination of stains will accomplish results that are impossible with Cyanine, C. I. No. 806, and Magdala Red, C. I. No. 857.

774. **Phloxine B,** 776. **Cyanosine,** 777. **Rose Bengale,** 778. **Phloxine B,** 779. **Rose Bengale B,** and 780. **Cyanosine B** are similar to Erythrosin in color and staining properties but they are more fugitive. The addition of potassium dichromate to the stain does not improve the fastness.

781. **Gallein** is a mordant dye, light 1; ½ gram in 100 ml. of either 1 per cent ammonium acetate or 1/10 per cent sulfuric acid.

Small invertebrates are mordanted for 30 minutes in 1 per cent ferric ammonium sulfate. After a rinse in distilled water, the material appears blue-black after one or two minutes in the stain at 50° C. With copper acetate as the mordant the color is Hematein-purple. Paraffin sections of animal tissues are stained violet with blue-black nuclei in 15 to 30 seconds at 50° C.

Paraffin sections of plant tissues mordanted in ferric alum and kept for five to ten minutes in the dye at 50° C. become reddish violet in the cytoplasm to blue-violet in the nuclei. With this dye in dilute sulfuric acid, the sections mordanted in iron alum take a blue nuclear stain. Whole mounts take on a Hematein-purple of excellent fastness. Blue-green algae are readily stained without the aid of a mordant.

783. **Cerulein MS,** Anthracene Green, Coerulein MS is a mordant dye, light 3 to 4; ½ gram in 100 ml. of 1 per cent ammonium acetate. This dye does not give a satisfactory stain on animal tissues. Vascular plant tissues mordanted in ferric alum become dull green in the stain at 50° C. In alkaline solutions of the dye blue-green algae are clear green.

Class XII. Acridine Colouring Matters

785. **Acridine Yellow R,** 2R is a basic dye, light 2; ½ gram in 95 ml. of distilled water, 5 ml. of 5 per cent ammonium alum, and ½ ml. of acetic acid. Unless the stain contains ammonium alum, the dye bleeds from sections mounted in balsam; more than the usual amount of time is needed for dehydration. The dye may be used in preference to Bismarck Brown, C. I. No. 331.

788. **Acridine Orange** is a basic dye, light 1 to 2; ½ gram in 95 ml. of distilled water, 5 ml. of 5 per cent ammonium alum, and ½ ml. of acetic acid. Plant tissues are stained a clear brown or dark orange of exceptional fastness. To prevent bleeding of the color in balsam, dehydrate the material slowly.

793. **Phosphine** is a basic dye, light 2 to 3. This dye is a by-product obtained in the manufacture of Basic Fuchsine. The stain is similar in color to Acridine Orange but not of equal fastness. The dye-solution decomposes on standing.

Class XIII. Quinoline Colouring Matters

A number of dyes with the property of color-sensitizing photographic plates belong here. Most of them are fugitive and expensive.

801. **Quinoline Yellow** is an acid dye, light 2; ½ gram in 95 ml. of distilled water, 5 ml. of ½ per cent potassium dichromate, and 1 ml. of 1 per cent sulfuric acid. On most plant tissues this stain is too light. A clear bright yellow is obtained more easily with the addition of potassium dichromate.

806. **Cyanine** is a basic dye, light 5. It is one of the most expensive as well as one of the most fugitive dyes. Nuclei are stained blue; the cytoplasm and cell walls have only a faint color. Algae in whole mounts are not stained evenly. The same color and staining reactions can be obtained with Capri Blue, C. I. No. 876.

Class XIV. Thiazole and Thiobenzenyl Colouring Matters

To this class belongs **Primuline,** C. I. No. 812, which is a fugitive acid dye that has little value for histological purposes.

Class XV. Indamine Colouring Matters

Indamine dyes are of no value as stains; they are intermediate products used in the manufacture of sulfur dyes.

Class XVI. Indophenole Colouring Matters

These substances are used in the manufacture of vat dyes.

Class XVII. Azine Colouring Matters

825. **Neutral Red,** Toluylene Red is a basic dye, light 3. Plant tissues stained a dull red in acid solutions become more intense red with the addition of ammonium alum. This dye is frequently used as a vital stain; the various parts of the living cell are differentiated in orange or red. It is also used as an indicator.

826. **Neutral Violet** is a basic dye, light 4. Plant tissues are stained a fugitive blue-violet. The dye solution decomposes on standing.

828. **Azocarmine GX** is an acid mordant dye, light 3 to 4. The dye dissolves in either 1 per cent ammonium acetate or in 5 per

cent ammonium alum. Invertebrates are orange-red after 15 minutes at 50° C. if ferric alum or copper acetate is used as the mordant. On most paraffin sections the color is too light. For a general stain of this color try Direct Fast Scarlet 4BS, C. I. No. 327.

841. **Safranine** A, G, O, Y is a basic dye, light 2 to 3; ½ gram in 95 ml. of distilled water, 5 ml. of 5 per cent ammonium alum, and ½ ml. of acetic acid. The letters Y (yellow) and G (gelb) refer to the bright scarlet shade; A and O indicate a product of the highest purity or of a greater dye content. Safranine is soluble in either water or alcohol.

Safranine, without the addition of an acid, stains slowly; the color washes out during dehydration. The presence of ammonium alum intensifies the color and reduces the time required for staining. Safranine and alum Haematoxylin is one of the favorite combinations for leaf, stem, and root sections; here the lack of contrast between red and purple gives blue or green dyes a preference to Haematoxylin. The material is stained in Safranine long enough to obtain an intense red, one to ten hours, and then, without destaining, the contrast color is added. The best counterstains for Safranine are: (1) Aniline Blue, C. I. No. 707, five minutes, (2) Niagara Sky Blue, C. I. No. 520, five to ten minutes, (3) Fast Green FCF, C. I. No. 670, ten to 15 minutes, or (4) alum Haematoxylin. After the material is stained in alum Haematoxylin a rinse in 0.01 per cent ammonium hydroxide will darken the purple pigment. A discussion of Flemming's triple stain, Safranine, Orange G, and Gentian Violet is given under Orange G, C. I. No. 27.

842. **Fuchsia,** Methylene Violet is a basic dye, light 3. Fuchsia has properties similar to Safranine; the color of stained tissues is more bluish and varies from fuchsia to reddish violet.

846. **Mauveine** is a basic dye, light 3. Mauveine, the first dye made from aniline, was discovered in 1856. Paraffin sections of plant tissues are stained blue-violet similar to the color obtained with Methyl Violet.

856. **Naphthyl Red** is a basic dye, light 5. Only the nuclei of mature plant cells are stained a fugitive red.

857. **Magdala Red** is a basic dye, light 3; 1/10 gram in 100 ml.

of 50 per cent alcohol and ½ ml. of acetic acid. The nuclei of parenchyma are stained bright red; the cytoplasm and cell walls, fuchsia.

860. **Acetin Blue R,** Induline alcohol soluble, is a basic dye, light 4. Plant tissues in paraffin sections are stained a dull light blue.

861. **Induline** water soluble is an acid dye, light 4 to 5. Plant tissues are stained light blue in acid solutions of the dye or black with the addition of potassium dichromate.

865. **Nigrosine** water soluble is an acid dye, light 3 to 4. Parenchyma is stained dull blue in an acid solution of the dye. With potassium dichromate present the cell walls are blue; the nuclei remain unchanged or yellow-brown.

Class XVIII. Aniline Black and Allied Colouring Matters

Class XIX. Oxazine Colouring Matters

876. **Capri Blue** is a basic dye, light 3; 1/10 gram in 95 ml. of distilled water, 5 ml. of 5 per cent ammonium alum, and ½ ml. of acetic acid. Plant tissues are stained blue or black. This dye may be used in preference to Cyanine, C. I. No. 806; it should counterstain well after Erythrosin, C. I. No. 773.

877. **Brilliant Cresyl Blue,** Brilliant Blue is a basic dye, light 3. Certain plants in whole mounts are stained blue but the color is not of uniform density.

883. **Gallocyanine** is a direct mordant dye. The pigment obtained at room temperature is fugitive, light 4. At 50° C. the color is intensified to a deep blue-black with an increase in fastness to that of the best dyes, light 1; 1/10 gram in 100 ml. of 1 per cent ammonium acetate.

Invertebrates are placed in 1 per cent ferric ammonium sulfate for 30 minutes. After a rinse in distilled water, the material, at 50° C., becomes a Hematein-purple in one or two minutes. Paraffin sections of animal tissues are violet within 30 seconds. Plant materials mordanted in ferric alum require five to ten minutes for whole mounts or 15 to 20 minutes on paraffin sections.

909. **Meldola Blue** is a basic dye, light 4 to 5. The dye is somewhat selective in that the nuclei are stained blue-black and the

cytoplasm blue-violet. With the addition of ammonium alum to the stain the color on plant tissues is more intense but it is still too fugitive to be of much value in histology.

913. **Nile Blue Sulfate** is a basic dye, light 2. The dye is slightly soluble in warm water but readily soluble in 50 per cent alcohol. It is unsuited for paraffin sections. Green algae which are usually overstained in basic dyes are colored a clear bright blue. The dye may be used as a vital stain.

917. **Alizarine Green G** is an acid mordant dye, light 1; 1/10 gram in 100 ml. of 1 per cent ammonium acetate. With 1 per cent ferric alum as a mordant followed by the stain for 30 minutes at 50° C. the resultant green is the clearest obtained with a mordant dye. Paraffin sections of animal tissues are stained green slightly tinted blue. Various plant tissues appear dull green with the ammonium acetate solution or blue in the stain containing sulfuric acid. Alizarin Green G may be used as a vital or as a fat stain.

Class XX. Thiazine Colouring Matters

920. **Thionine** is a basic dye, light 3; 1/10 gram in 95 ml. of distilled water, 5 ml. of 5 per cent ammonium alum, and ½ ml. of acetic acid. Plant tissues are stained dark blue to violet. In those examples of contrast staining where Methyl Violet does not differentiate well, Thionine may give the desired results. Blue-green algae are stained purple in alkaline solutions of the dye but this color is not fast in glycerin. As a counterstain, Thionine may be used after Acid Fuchsine, C. I. No. 692, Biebrich Scarlet, C. I. No. 280, or Orange II, C. I. No. 151.

922. **Methylene Blue** is a basic dye, light 2 to 3; ½ gram in 95 ml. of distilled water, 5 ml. of 5 per cent ammonium alum, and ½ ml. of acetic acid. Unna's polychrome Methylene Blue is Methylene Blue ripened in a solution containing potassium carbonate. The color of the dye is fugitive unless an acid and ammonium alum are added to the dye solution. This dye has been used more than any other basic blue dye for staining bacteria, blood, and animal tissues. Because blood stains provide only temporary mounts, the technique has been omitted. New Methylene Blue is more soluble; for plant tissues it is a better stain than Methylene Blue.

923. **Methylene Azure,** Azure I is a basic dye, light 3; 1/10 gram in 95 ml. of distilled water, 5 ml. of 5 per cent ammonium alum, and ½ ml. of acetic acid. Methylene Blue in solution changes to Methylene Azure by oxidation. Methylene Azure includes a mixture of Azure A and Azure B. Azure C is another oxidation product of Methylene Blue. Azure II is a mixture of equal parts of Methylene Blue and Methylene Azure. All of these dyes are polychromatic, a property which makes them valued for blood stains. The preparation of blood stains includes the oxidation of Methylene Blue and the addition of eosin.

By staining a section of pine wood in Methylene Azure or Methylene Blue for 15 minutes, the torus becomes bright red while the walls of the tracheids are blue; by staining for a longer time only a blue is obtained. Unfortunately, these colors are not fast. The addition of acids, alkalies, or ammonium alum greatly affects the stain. Acid solutions of the dye react slowly with a better differentiation than neutral solutions; an alkaline solution will stain 25 times more rapidly than an acid solution. The addition of ammonium alum intensifies the color in either dye-solution.

924. **Methylene Green** is a basic dye, light 3; ½ gram in 95 ml. of distilled water, 5 ml. of 5 per cent ammonium alum, and ½ ml. of acetic acid. In acid solutions of the dye, plant tissues are stained green to blue-green. The intensity of the color is reduced but more clear in differentiation if ammonium alum is present in the stain. This dye is the best of the green basic dyes; it provides a good counterstain after Acid Fuchsine, C. I. No. 692, Biebrich Scarlet, C. I. No. 280, or Crocein Scarlet MOO, C. I. No. 252.

925. **Toluidine Blue O** is a basic dye, light 3. Plant tissues become blue similar in most respects to Methylene Blue. It may be used as a substitute for Thionine, C. I. No. 920, or Azure A, C. I. No. 923.

926. **Rhoduline Blue GO,** Thionine Blue GO is a basic dye, light 4. Plant tissues are stained blue or blue-green that is similar to but not so fast as Methylene Blue, C. I. No. 922.

927. **New Methylene Blue,** N, NSS is a basic dye, light 2 to 3; ½ gram in 95 ml. of distilled water, 5 ml. of 5 per cent ammonium

alum, and ½ ml. of acetic acid. New Methylene Blue is a constituent of several triple and quadruple stains which are described under the separate acid dyes. These combinations of stains can be used on sections of embryonic animal tissues. For the general staining of plant tissues New Methylene Blue gives better results than either Methylene Blue or Methylene Azure. It may be used as a contrast stain after Acid Fuchsine, C. I. No. 692, Biebrich Scarlet, C. I. No. 280, Crocein Scarlet MOO, C. I. No. 252, Orange II, C. I. No. 151, or Sun Yellow, C. I. No. 620.

931. **Brilliant Alizarin Blue** is a basic dye, light 3 to 4. This stain is darker than New Methylene Blue. The stain is not so good with the addition of ammonium alum; with alkalies very little color is produced.

Class XXI. Sulfur and Sulfide Colouring Matters

Many of the sulfur dyes are not classified in the *Colour Index* because their chemical composition at present is unknown. There are no bright red sulfur dyes.

948. **Sulfur Yellow,** or **Sulfur Orange,** and 949. **Sulfur Brown 4G** differ very little from Sulfur Bordeaux.

956. **Sulfur Direct Blue,** Sulfogene Direct Blue BRS (Du Pont) is a direct dye, light 2; 1/10 gram in 95 ml. of 1 per cent sodium sulfide and 5 ml. of 4 per cent sodium hydroxide. Blue-green algae are not stained so intensely nor so brightly as with Sulfur Brilliant Blue. The details of cell structure are presented very clearly.

957. **Sulfur Brilliant Blue,** Sulfindone Brilliant Blue CG (NAC), Sulfogene Brilliant Blue 6BS (Du Pont), and Sulfogene Brilliant Blue 3GCF (Du Pont) are direct dyes, light 2; 1/10 gram in 95 ml. of 1 per cent sodium sulfide and 5 ml. of 4 per cent sodium hydroxide. Because the stain does not keep over a day, it must be made as needed. These are the best blue sulfur dyes; the color is nearly violet on algae mounted in glycerin. Algae are placed in the stain one to three hours at 50° C., rinsed with tap water, and then oxidized to a deeper shade as soon as the moist plants are exposed to air, for 15 minutes. Invertebrates such as *Obelia,* or jelly-fish

of large size are stained within 15 minutes. All of these materials are dehydrated from 10 per cent glycerin.

1006. **Sulfur Green,** Sulfogene Green 2B (Du Pont), Sulfogene Brilliant Green 2G (Du Pont), and Sulfur Green 3G conc. (NAC) are direct dyes, light 2; 1/10 gram in 100 ml. of 1 per cent sodium sulfide and 1 ml. of 4 per cent sodium hydroxide. The 3G shade gives a bright green; the 2B, a blue-green at 50° C. on blue-green algae in three to ten hours, on invertebrates in 30 minutes.

1012. **Sulfur Bordeaux,** Sulfogene Bordeaux BRN (Du Pont), and Sulfur Bordeaux BCF (NAC) are direct dyes, light 2; 1/10 gram in 95 ml. of 1 per cent sodium sulfide and 5 ml. of 4 per cent sodium hydroxide. At 50° C., plant tissues are stained in 12 to 24 hours, invertebrates in 30 minutes. The oxidation treatment is unnecessary for Sulfur Bordeaux.

Class XXII. Hydroxyketone Colouring Matters

These dyes are used in the color printing of wool.

Class XXIII. Anthraquinone Colouring Matters

1027. **Alizarin** is an acid mordant dye, light 4 to 3; ½ gram in 100 ml. of distilled water and 1 ml. of 4 per cent sodium hydroxide. The dye is insoluble in water, slightly soluble in alcohol, and blue-violet in dilute alkalies. Upon the addition of an acid to the alkaline stain, a yellow precipitate is formed. The dye produces a wide range of colors depending on the metallic salt used as a mordant, the alkalinity of the stain, and the temperature of application. A few of the mordants with the accompanying color lakes are: aluminum, orange to red; copper, carmine; antimony, brown; chromium, bordeaux; uranium, blue-violet; and iron, blue-black to black. All of the lakes are darker when developed at 50–60° C.

The stain is easily applied to invertebrates of any size. The technique includes a mordant for 30 minutes; after a rinse in distilled water, Alizarin is applied at 40–50° C. for five minutes. The best colors develop after: ammonium alum, orange-red to red; copper acetate, carmine; or ferric alum, dark red at room temperature to blue-black at 50° C. Paraffin sections of earthworm are dull red

in one minute when ferric alum is used as the mordant but the color is not clear and the differentiation is not satisfactory.

A good brick red may be obtained on plant tissues with the following procedure: 15 minutes in turkey red oil solution, rinse in distilled water, 15 minutes in a saturated solution of ammonium alum, rinse in water, five minutes in lime water, rinse, and 12 to 24 hours in an alkaline solution of Alizarin. Turkey red oil is sulfonated castor oil. To 100 ml. of distilled water is added 1 ml. of turkey red oil and two or three drops of ammonium hydroxide to give a clear solution.

1034. **Alizarin Red S,** Alizarin Sodium Sulfonate is a direct mordant dye, light 4; ½ gram in 95 ml. of distilled water, 5 ml. of 5 per cent ammonium alum, and ½ ml. of acetic acid. After a plant section has been in the stain 12 to 24 hours, it is rinsed in distilled water and dipped into turkey red oil solution in order to develop the orange-red pigment.

1037. **Purpurin** is an acid mordant dye, 3 to 4; 1/10 gram in 80 ml. of 5 per cent ammonium alum, boil, cool, filter, and add 20 ml. of 95 per cent alcohol which serves as a preservative. Plant tissues are stained a brilliant red within 24 hours. The dye counterstains easily with a large number of basic blue, violet, or green dyes. Unfortunately, this red is fugitive.

1048. **Acid Alizarin Blue GR** is a direct mordant dye, light 2. The dye dissolves either in 1 per cent ammonium acetate or in 5 per cent ammonium alum. The stain is not active until heated to 50° C.; at this temperature, mordanted sections of plant tissues are stained bright blue. It is difficult to obtain a satisfactory preparation because a precipitate separates from solution soon after the stain is heated.

1049. **Acid Alizarin Green G** is a direct mordant dye, light 1; 1/10 gram in 100 ml. of 1 per cent ammonium acetate. Blue-green and green algae or fungi mordanted in ferric alum become clear green within five to ten minutes at 50° C. The dye is not so good for invertebrates in whole mounts. On paraffin sections of animal tissues mordanted in 1 per cent ferric alum, light green is produced in 30 seconds and clear green in one minute at 50° C.

1050. **Alizarin Cyanine R** is an acid mordant dye. A solution of this dye is not stable and, on heating, a reddish precipitate prevents the development of a clear stain.

1051. **Alizarin Cyanine G** is a direct mordant dye, light 1; 1/10 gram in 100 ml. of 1 per cent ammonium acetate. Sections of plant materials that are mordanted in ammonium alum are stained a clear Hematein-purple. Objects that are mordanted in ferric alum are red-violet to blue-violet within five to ten minutes at 50° C. Blue-green algae are stained purple in alkaline solutions. When ferric alum is used as a mordant, paraffin sections of animal tissues or whole mounts of invertebrates are blue-violet in two to three minutes at 50° C.

1054. **Alizarin Sapphire BN** (NAC) is a direct mordant dye, light 2. Sections mordanted in ferric alum show only a trace of color at 50° C. in the ammonium acetate strain; in the sulfuric acid solution of the dye, nuclei are light blue.

1067. **Alizarin Blue S** is a mordant dye, light 3. The dye dissolves in slightly alkaline 50 per cent alcohol but the blue pigment separates from solution either in the presence of acids, alkalies, or upon heating.

Class XXIV. Anthraquinone Vat Colouring Matters

1101. **Carbanthrene Jade Green** (NAC).
1104. **Carbanthrene Violet 2R** (NAC).
1113. **Carbanthrene Blue GD** (NAC).
1131. **Ponsol Red 5GK** (Du Pont).
..... **Ponsol Red AFF** (Du Pont).
..... **Carbanthrene Brilliant Orange RK** (NAC).
1162. **Carbanthrene Red BN** (NAC).
..... **Sulfanthrene Red 3B** (Du Pont).

A few of the cold vat dyes may be applied to blue-green algae at 50–60° C. The preparation of the stain is described under Indigo, C. I. No. 1177.

Class XXV. Arylidoquinone Vat Colouring Matters

Class XXVI. Indigoid Colouring Matters

1177. **Indigo** is a vat dye, light 1; 1/10 gram of the dye is

worked into a paste in a casserole with 5 ml. of 35 per cent sodium hydroxide, to which is added slowly 45 ml. of hot water, and then, with continuous stirring, ½ gram of sodium hydrosulfite powder. The vat may be colorless or it may be different from the original dye; Indigo changes from blue to yellow-green in the vat. After five to ten minutes at 60° C., the material is taken out of the stain and rinsed in warm water. The stained object should be the same color as the original dye. Pigments obtained from bright orange or red dyes disappear from blue-green algae soon after the algae are placed in glycerin, but jade green, violet, and indigo are fast colors.

1180. **Indigo Carmine,** Indigotine is a direct dye similar in color to Niagara Sky Blue 6B; ½ gram in 95 ml. of distilled water, 5 ml. of 5 per cent sodium sulfate, and ½ ml. of acetic acid. The stain gives an indigo on sections of animal tissues within ten minutes, on plant tissues in one to 24 hours. An important property of the dye is its selectivity in staining nuclei, and its delicate shading of embryonic or plant meristematic tissues. These minute differences in muscular, vascular, nervous, and digestive tissues are evident after a triple or quadruple stain including: (1) a mixture of nine parts Sun Yellow and one part Acid Fuchsine for ten minutes followed by Indigo Carmine for five to ten minutes; or (2) a mixture of ten parts Sun Yellow, five parts Orange II, and one part Acid Fuchsine, followed by Indigo Carmine.

Chapter VII

BIOLOGICAL STAINS

B. Natural Organic Dyestuffs

Since prehistoric times numerous natural dyes have been used in the dyeing of fabrics. It was not until the discovery of the art of mordanting, however, that any considerable advance in dyeing became possible. The mordants at first employed consisted of the naturally occuring sulfates of iron and aluminum.

1239. **Carmine,** Carminic Acid, or Cochineal is an acid mordant dye, light 3 to 4. Cochineal is extracted with water containing ammonium alum from the tropical insect, *Coccus cacti,* reared on cactus plants in Mexico. The purified red pigment is known as Carmine. The active coloring principle of this dye is Carminic Acid, which, upon the addition of ammonium alum, potash alum, borax, sodium stannate, or sulfuric acid becomes soluble in water.

The stain is prepared either from the dry powdered cochineal bugs or from the purified Carminic Acid.

1. A saturated stain contains 0.1 gram of Carminic Acid or the extract from 0.5 gram of powdered bugs in 100 ml. of 0.2 per cent sulfuric acid at 50° C.; several hours is necessary to effect complete solution. Filter the hot liquid and add 20 per cent alcohol by volume. Invertebrates or chick embryos are placed in a tin mordant for 30 minutes; they are stained a brilliant scarlet within 30 minutes at 50° C. This solution is unsatisfactory for paraffin sections unless the material is first stained in bulk.

2. Dissolve 0.1 gram of Carminic Acid or extract 0.5 gram of cochineal bugs in 100 ml. of 1 per cent ammonium acetate in 20 per cent alcohol. A brilliant carmine is obtained on whole mounts of invertebrates with a 30-minute mordant in 5 per cent ammonium alum and a stain for 30 minutes at 50° C. This ammonium acetate solution may be used for paraffin sections although the red developed in 24 hours is not so good as the color obtained by staining in bulk.

3. Carmine dissolves in a solution of 5 per cent ammonium alum

in 20 per cent alcohol. The alum stain yields lavender or fuchsia with aluminum, tin, or copper mordants; blue-black to black with uranium or iron salts. The alcohol added to the stain serves as a preservative.

1242. **Litmus,** Archil, Orcein is a dye extracted from different lichens. Its principal value is that of an indicator.

1243. **Brazilin,** Brazil Wood is an acid mordant dye, light 5. The tropical redwood *Caesalpinia,* family Leguminosae, contains Brazilin which is soluble in water or alcohol, and is changed on oxidation to a red solution of Brazilein. Plant tissues are stained carmine to dull brown but the color is of low fastness.

1246. **Haematoxylin,** Hematein is an acid mordant dye, light 2 to 4. The dye is obtained from the wood of *Haematoxylon campechianum,* family Leguminosae, indigenous to Mexico and Central America but cultivated in the West Indies. When freshly cut the wood is colorless; upon exposure to air, the surface of the wood becomes reddish brown with the development of Haematoxylin from a glucoside. After chipping the wood, the dye is extracted with boiling water in open pans or with steam under pressure in closed vessels. The extract is concentrated to a liquor or a solid. On further treatment, the crude product yields the light brown crystals of Haematoxylin.

The fastness of Haematoxylin differs with (1) the various metallic salts used as mordants, (2) the kind of staining solution, and (3) the temperature of application. The color obtained with iron Haematoxylin has a fastness of 3 to 4; of alum Haematoxylin, 3. By using iron alum as a mordant and the stain ripened in dilute alcohol, the color developed at 50° C. has a fastness of 2, almost the equal of the best coal-tar dyes.

Directions for making the solution as originally given by J. Delafield in *Zeit. Wiss. Mik.* 2: 288 (1885) are: "To make 600 cc of the solution, take 400 cc saturated aqueous solution of ammonium alum and add to this 4 grams of crystallized Haematoyxlin dissolved in 25 cc strong alcohol. This at first produces a light violet or sometimes a dirty red color, but on exposure to the light in an unstoppered bottle the color deepens and a light precipitate forms.

After standing for three or four days exposed to the air and light, the solution is filtered and 100 cc each of glycerin and methyl alcohol are added. The solution is now allowed to stand until the color is sufficiently dark and is then filtered and kept in a tightly stoppered bottle. This solution must be considerably diluted with water before using." (N. Y., April 18th, 1885.)

This original formula of Delafield (as given above) contains a number of errors. It is impossible to dissolve four grams of Haematoxylin in 25 ml. of strong alcohol because the solubility of the dye is approximately $\frac{1}{2}$ per cent. Therefore, if only the dissolved portion of the dye is added to the alum solution, about 95 per cent of the crystals remain unused. Since Haematoxylin is slightly more soluble in ammonium alum than in alcohol, it is unnecessary to use alcohol as a solvent. As soon as alcohol is added to aqueous ammonium alum, the few crystals first thrown out of solution serve as centers for the rapid growth of larger crystals. Ammonium alum is soluble in water but insoluble in alcohol. At the time the stain is filtered, a considerable quantity of the dye and half of the alum remain undissolved. Since it has long been the practice of technicians to employ concentrated stains, a saturated solution of Haematoxylin in ammonium alum, then, is evidently what Delafield wished to obtain.

Taking into consideration the characteristic properties of Haematoxylin as a dye, a saturated stain containing ammonium alum is made in accordance with the formula given below.

Alum Haematoxylin (aluminum Haematoxylin).

Distilled water	160 ml., the solvent
Alcohol, 95%	40 ml., the preservative
Ammonium alum ..	10 grams, the mordant
Haematoxylin	1 gram, optimum solubility

This staining solution is ripened in three days if kept at 60° C. in a liter Erlenmeyer flask plugged with cotton. After having been ripened, the stain is filtered. By adding 20 ml. of glycerin, which retards the oxidation of the stain, the keeping qualities are improved. Should methyl alcohol instead of ethyl alcohol be added

to the solution, the color produced by the stain is more reddish violet.

Iron Haematoxylin is ½ per cent Haematoxylin in water or dilute alcohol. The mordant, 1 to 4 per cent ferric ammonium sulfate, is applied separately. Heidenhain gives detailed directions for the preparation and use of the stain (Martin Heidenhain, "Noch einmal über die Hämatoxylinfarben," *Zeit. Wiss. Mik.* 13: 186–199 (1896)). He reports the application of iron Haematoxylin by Benda on nervous tissue, by Bütschli on cytoplasm, and by Sobotta on tissues fixed in vapors of osmic acid. Heidenhain recommends 2½ to 4 per cent ferric ammonium sulfate, because weaker solutions decompose quickly. Paraffin section on slides are placed vertically in the mordant six to twelve hours. The stain is given as Weigert's solution containing 1 gram of Haematoxylin in 10 per cent alcohol. Four weeks are needed for the ripening, and then, the stain is diluted with an equal volume of distilled water. Tissues are stained 24 to 36 hours, rinsed, differentiated in 2½ per cent ferric alum, and washed in running water 10 to 15 minutes.

Iron Haematoxylin is applied in two solutions: the mordant ferric ammonium sulfate and an aqueous stain.

A. The mordant.	
Distilled water	100 ml.
Ferric ammonium sulfate, clear lavender crystals	1 gram, or as much as 4 grams
B. The stain.	
Distilled water	160 ml.
Alcohol, 95%	40 ml., the preservative
Haematoxylin	1 gram

The stain is ripened, in a liter Erlenmeyer flask plugged with cotton, at 60° C. for two days. The 1 per cent ferric alum should not be kept more than three days because a trace of sulfuric acid, which appears in dilute solutions of ferric alum on standing, produces a greenish Hematein pigment.

A definite correlation exists between the fixatives, tissues, and stains. Using alum Haematoxylin, a tissue fixed in formalin

presents a general stain while the material taken from chrom-acetic yields a nuclear stain. Similar results are obtained with iron Haematoxylin. Should sublimate-acetic be used as the fixative, the results differ: a general stain is obtained whenever the material is left in the dye only long enough to provide the color pigment; by overstaining in Haematoxylin and destaining in iron alum, the result is a nuclear stain.

Staining with Haematoxylin. The procedure in which the reaction is checked as soon as sufficient color has been produced is known as the progressive method of staining. Alum Haematoxylin is a progressive stain. The ripened solution stains animal tissues within five minutes, plant tissues in 15 to 30 minutes. Should the material be overstained in a few minutes, the solution may be diluted with water.

The regressive method of staining is followed when the dye accumulates to excess, whereupon the surplus pigment must be removed later with a suitable solvent or reducing agent. Iron Haematoxylin is a regressive stain. For a rapid schedule, animal tissues are mordanted in 1 per cent ferric alum 15 minutes, rinsed, stained in ripened Haematoxylin one minute, differentiated in 0.25 per cent ferric alum for five minutes, rinsed, left in 0.01 per cent ammonium hydroxide three minutes, and rinsed with distilled water. With a longer schedule, the material is left in 2 to 4 per cent ferric alum 12 to 24 hours, rinsed, stained 24 hours, differentiated in 1 per cent ferric alum, rinsed, left in 0.01 per cent ammonium hydroxide three minutes, and rinsed with distilled water. Plant tissues are mordanted in 1 per cent ferric alum 30 minutes, rinsed, stained in Haematoxylin three minutes, differentiated in 0.25 per cent ferric alum five to ten minutes, rinsed, left in 0.01 per cent ammonium hydroxide three minutes, and rinsed with distilled water. A slower schedule requires 24 hours in the mordant and 24 hours in the stain; the blue-black pigment is so fast in root tips there is no evidence of fading after twenty years.

As a rule, a tissue prepared for cytological studies does not require a counterstain. Under low power objectives, a trace of color in the cytoplasm aids in bringing out the third dimension of the

image. Naphthol Green B is well suited as a counterstain because generally it is applied with iron alum, is fast to light, and does not extract any of the Hematein. After the sections have been stained in iron Haematoxylin and differentiated in iron alum, the material is rinsed a few seconds in distilled water and counterstained in Naphthol Green B for five minutes.

1249a. **Chlorophyll,** Carotene (Carotin), and Xanthophyll are naturally occurring pigments, light 4. In microscopic plants, the green pigment may be fixed with the aid of copper acetate; this fixation is probably a replacement of the magnesium in chlorophyll by copper.

Fixative, methyl alcohol-formalin-acetic

Methyl alcohol	100 ml.
Formalin, 40% commercial	100 ml.
Acetic acid, 10%	100 ml.
Copper acetate	1 gram

Because chlorophyll is soluble in alcohol, fixed materials for permanent mounts in their natural colors are washed in water and dehydrated from 10 per cent glycerin.

C. Inorganic Colouring Matters

1288. **Prussian Blue,** Berlin Blue, Chinese Blue is ferric ferrocyanide, light 1 to 2. The pigment may be used as a stain for whole mounts of algae, fungi, or invertebrates. The material is placed first in 3 per cent potassium ferrocyanide for 30 minutes, rinsed thoroughly with distilled water, and then covered with a solution of 1 per cent ferric ammonium sulfate. Should the color be too light, staining may be repeated until the desired shade is obtained. The 10 per cent glycerin used for dehydration should contain a few drops of $\frac{1}{2}$ per cent potassium dichromate so that the ferric ferrocyanide does not change to the soluble and colorless ferrous ferrocyanide. Prussian Blue is stable in either balsam or glycerin.

INDEX

Colour Index Numbers

Iron Haematoxylin, 1246
Isamine Blue 6B, 710
Janus Green B, 133
Janus Red B, 266
Lanacyl Violet B, 207
Lanacyl Blue BB, 210
Light Green SF, 670
Litmus, 1242
Magdala Red, 857
Magenta, 677
Malachite Green, 657
Martius Yellow, 9
Mauvine, 846
Meldola Blue, 909
Metanil Yellow, 138
Methyl Blue, 705, 706
Methyl Eosin, 769
Methyl Green, 684
Methyl Orange, 142
Methyl Red, 211
Methyl Violet B, 2B, 680
Methyl Violet 5B, 6B, 683
Methyl Violet 10B, 681
Methylene Azure, 923
Methylene Blue, 922
Methylene Green, 924
Methylene Violet, 842
Mikado Yellow G, 622
Naphthol Green B, 5
Naphthol Yellow S, 10
Naphthyl Red, 856
Naphthylamine Brown, 175
Naphthylamine Red, 184
Naphthylamine Yellow, 9
Neutral Red, 825
Neutral Violet, 826
New Fuchsine, 678
New Magenta, 678
New Methylene Blue, N, NSS, 927
Niagara Sky Blue, 520
Niagara Sky Blue, 6B, 518
Night Blue, 731
Nigrosine, water soluble, 865
Nile Blue Sulfate, 913
Opal Blue, 689
Orange G, 27
Orange R, 161
Orange II, 151
Orange III, 142
Orange IV, 143
Orcein, 1242
Para Red, 44
Pararosaniline, 676
Patent Blue A, 714
Phenolphthalein, 764
Phloxine B, 774, 778
Phosphine, 793
Picric Acid, 7
Ponceau G, 2G, 28
Ponceau R, 2R, 79
Ponceau 3R, 80
Ponsol Golden Orange RRT, 1097
Ponsol Red 5GK, 1131
Pontachrome Brown MW, 101
Pontachrome Orange R, 415
Pontacyl Carmine 6B, 57
Pontacyl Carmine 2G, 31
Pontamine Fast Pink BL, 353
Pontamine Fast Scarlet 4BS, 327
Primuline, 812
Prussian Blue, 1288
Purpurin, 1037
Pyronine B, 741
Pyronine 2G, 739
Quinoline Yellow, 801
Red Violet 5R, 679
Rhodamine B, 749
Rhodamine G, 750
Rhodamine 6G, 752
Rhoduline Blue GO, 926
Rose Bengale, 777
Rose Bengale B, 779
Roseine, 677
Rosolic Acid, 724
Rubine S, 692
Safranine A, G, O, Y, 841